The Deubiquitylation Enzymes MATH-33, USP-47, and USP-46 are Required for Polarity Establishment in *Caenorhabditis elegans*

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The *Caenorhabditis elegans* one-cell embryo polarizes in response to a cue from the paternally donated centrosome, and asymmetrically segregates cell fate determinants that direct subsequent developmental events. I found that genes encoding MATH-33 and USP-47, two putative deubiquitylating enzymes (DUBs), are required for polarization of one-cell embryos. Maternal loss of MATH-33 and USP-47 leads to the inability of the embryo to correctly establish and maintain asymmetry as defined by the distributions of the posterior and anterior polarity proteins PAR-2 and PAR-3. Both the cortical flow that establishes polarity and positioning of the centrosome are affected by loss of these deubiquitylation genes, suggesting that the cause of polarity defects is due to a lack of proper polarity initiation. Because these DUBs are homologous to two members of a group of DUBs that act in fission yeast polarity, I tested additional members of that family and found that another *C. elegans* DUB gene, *usp-46,* also contributes to polarity. My finding that deubiquitylating enzymes required for polarity in *Schizosaccharomyces pombe* are also required in *C. elegans* raises the possibility that these DUBs act through an evolutionarily conserved mechanism to control cell polarity.

Biographical Sketch

Richard James McCloskey was born in Philadelphia, Pennsylvania in June 1984 to Linda and Richard McCloskey. Rich grew up with his immediate family which included his sister Lori Ann Navin and his brother Sean Francis McCloskey. Rich first became curious about biology through popular media. His first lesson in genetics involved learning that fictional turtles exposed to gamma radiation would receive DNA damage that could result in fantastically positive phenotypic changes that include the ability to become a ninja. He would later learn that real mutations are not nearly so spectacular. Shortly after Rich's ninth birthday in the summer of 1993 he saw the movie Jurassic Park based on the book by Michael Crichton. He acquired the book *Jurassic Park* from his grandmother Anna Lynch, read it during the summer, and continued to read similar fictional works by Crichton and others that featured biology, medicine, and technology. Rich attended Roman Catholic High School in Philadelphia where he met several of his best friends. Due to his interest in science, Rich chose microbiology as a major and attended the University of the Sciences in Philadelphia. Rich came to Cornell University to study Biochemistry, Cell, and Molecular biology in the Fall of 2006 and joined the Kemphues lab in 2007 to perform his thesis work. Rich met his wife Laurie in Ithaca on August 15th, 2009 and asked her to marry him 364 days later overlooking Niagara Falls.

To my family.

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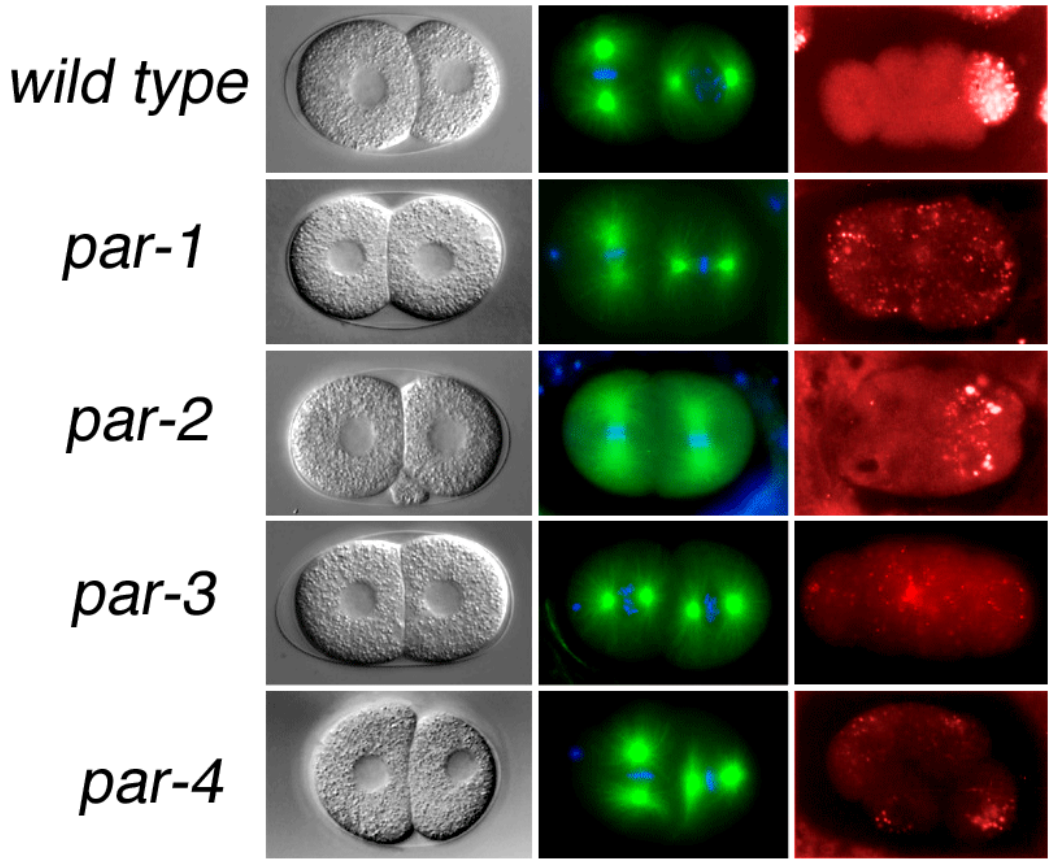
Chapter One. Introduction.

Part One. Polarity in *C. elegans.*

1.1. Introduction to the *pars*.

The *C. elegans* anterior-posterior axis is determined in the P0 blastomere of newly fertilized zygotes. This axial determination step is required to create the P1 and AB lineages that will undergo subsequent invariant divisions to form a worm (Sulston, Schierenberg, White, & Thomson, 1983). This polarization process is mediated by many components of the cell, but one specific set of genes plays a very important role, and these are called the *pars.* The *par* genes (standing for *par*titioning defective) were named based on their phenotypes, which include defects in partitioning cytoplasmic components, including cell fate regulators (Figure 1.1) (Kemphues et al., 1988; Morton et al., 2002; Watts et al., 1996). One such cytoplasmic component is P granules, which are large liquid-like aggregates of proteins and RNAs that normally segregate with the germline cell lineage (P lineage) of *C. elegans* (Strome and Wood, 1982; Strome and Wood, 1983; Brangwynne et al., 2009). Some of the PAR proteins localize specifically to either the anterior (PAR-3, PAR-6, and PKC-3) or posterior of the embryo (PAR-1, PAR-2, and LGL-1), suggesting that their function depended on their localization (Boyd et al., 1996; Etemad-Moghadam et al., 1995; Guo and Kemphues, 1995; Hung and Kemphues, 1999; Beatty et al., 2010). Indeed, mutations that perturb the ability of PAR-2, PAR-3, and PAR-6 to localize to the cortex impair their functionality (Hao et al., 2006; Li et al., 2010; Li, Kim, et al., 2010).

Figure 1.1.



The *par* genes display loss of asymmetric cell size, mitotic spindle orientation, and the mis-segregation of cytoplasmic components. Shown are wild type embryos and four *par* mutations. The first column shows the AB blastomere, and P1 blastomer (left and right) and all embryos are oriented with the anterior on the left. Shown in green are microtubules at the time AB and P1 set up mitotic axes, and shown in red are P granules at the four-cell stage in which granules normally localize to P2.

As a result of these unique localizations to either the posterior or the anterior, the PARs themselves can be used as precise markers to examine polarization. Most of the PAR proteins also have conserved homologs in many eukaryotes including mammals and the fruit fly *Drosophila melanogaster* (reviewed in Goldstein and Macara, 2007). In the other animal models, the PARs also govern cell polarity in various cell types of interest including epithelial cells and neurons (reviewed in Goldstein and Macara, 2007). Further studies in *C. elegans* have revealed that the *pars* have active functions in both establishing and maintaining polarity in the one cell embryo. I explain below the role of the *pars* in the establishment and maintenance phases of polarity in greater detail.

1.2. Polarity Establishment Results from a Centrosome Derived Cue.

How embryos polarize is a well characterized but complex process. PARs and cell fate determinants only become asymmetric if one-cell embryos undergo a distinct polarity establishment phase (Cuenca et al., 2003). Above all, this process depends on the one-cell embryo's centrosome.

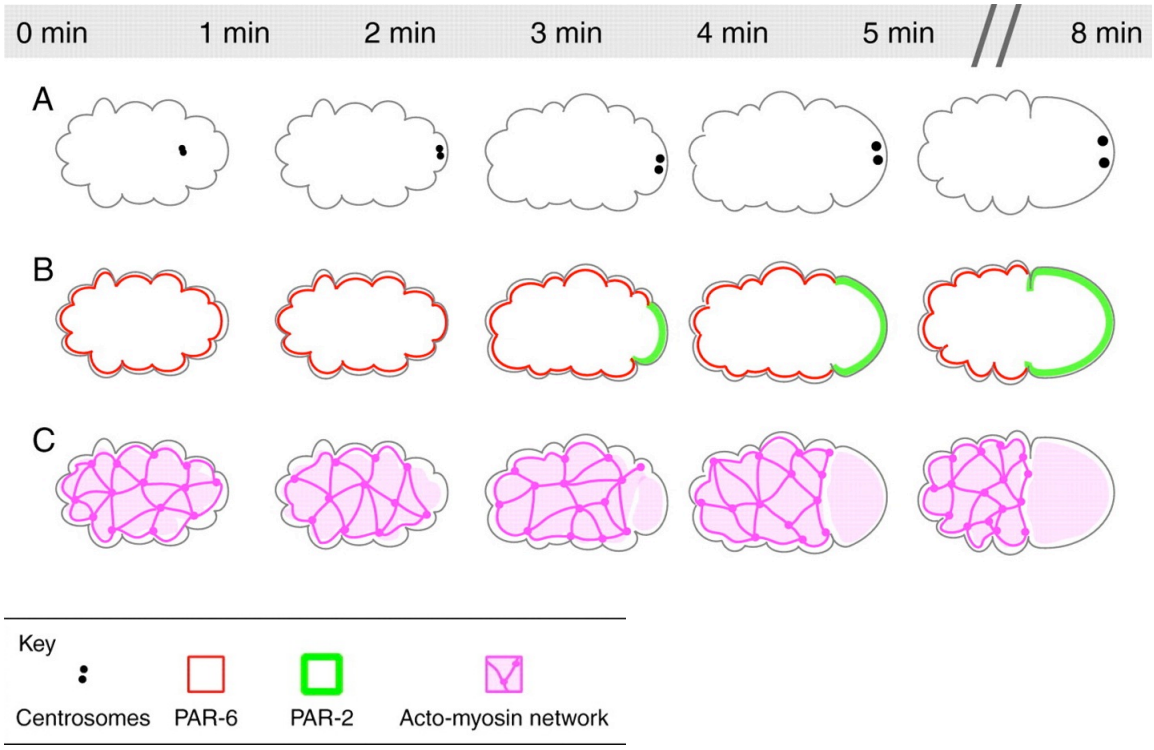
C. elegans oocytes are oval shaped, and generally lack cell asymmetry of any kind except that the maternal DNA, paused in meiosis, is localized near the membrane of the oocyte at the pole distal to the spermatheca (Johnston & Dennis, 2011). Typically, the end of the oocyte that is distant from the maternal DNA enters the spermetheca first where it is immediately fertilized (Johnston & Dennis, 2011). The side of zygote that the sperm enters usually becomes the posterior pole of the

embryo (B Goldstein & Hird, 1996). The most important sperm component that is contributed is the centriole. After fertilization the centriole matures into the centrosome, localizes to the posterior cortex, and initiates asymmetry (Cuenca et al., 2003; Cowan and Hyman, 2004; Munro et al., 2004; Tsai and Julie Ahringer, 2007; Cowan and Hyman, 2007). The protein components of the centrosome that are required for this cue are unknown. However, different hypotheses exist, with some investigators arguing that microtubules that extend from the centrosome are responsible for the cue (Wallenfang and G Seydoux, 2000; Tsai and Julie Ahringer, 2007; Motegi et al., 2011). Others believe that a centrosome–associated protein or proteins that emanate or diffuse from the centrosome are required to initiate the cue to break polarity (Bienkowska & Cowan, 2012; Cowan & Hyman, 2004; Sumiyoshi & Sugimoto, 2012).

The centrosome-based symmetry breaking event consists of at least two pathways. The major polarity cue depends on centrosome proximity to the embryo cortex (Cowan & Hyman, 2004; Lyczak et al., 2006). In the current best model for polarity initiation the centrosome signals the cortex and causes a local inactivation of the actomyosin network. The consequences of the cue are that ECT-2 (a RHO-GEF), and RHO-1 have a reduced presence at the cortex near the centrosome (Motegi and Sugimoto, 2006; H. Kim, K. Kemphues, Unpublished data). One model is that ECT-2 could be locally down-regulated at the cortex near the centrosome, and as a result RHO-1 loses the ability to be activated by ECT-2. The resulting loss of RHO-1 activity near the centrosome causes a reduction of contractility in the local actomyosin network. As a result of this local reduction the actomyosin contractility

at other parts of the cortex causes a cortical flow that allows actomyosin and other proteins to move from the posterior into the anterior (Figure 1.2) (Cowan & Hyman, 2007). This flow creates inherent asymmetries in the structure of the cortex at the anterior and the posterior, and these differences may help to recruit the posterior PARs PAR-1, PAR-2, and LGL-1 (Beatty, Morton, & Kemphues, 2010; Cheeks et al., 2004). In the auxiliary mechanism, microtubules contribute to polarity establishment by recruiting the posteriorly localized protein PAR-2 (Motegi et al., 2011; Zonies, Motegi, Hao, & Seydoux, 2010).

Figure 1.2



Establishment of polarity depends on a signal from the centrosome and the contractility of the actomyosin cytoskeleton. Shows are diagrams of embryos over time. The centrosome localization to the cortex is an early event that occurs before a break in asymmetry in the embryo. Afterwards, myosin (pink) and the anterior PARs (red) leave the posterior and posterior PARs (green) accumulate at the posterior cortex. This figure has been modified from (Cowan & Hyman, 2007) and used with permission for this thesis only, and should not be cited directly as the original work of this thesis.

The known primary establishment pathway depends on centrosome function and proximity to the cortex. For example, abrogating centrosome maturation by loss of *spd-2,* a gene required for centrosome maturation, prevents the establishment of polarity (O'Connell, Maxwell, & White, 2000). Furthermore, studies in which the centrosome spends less time near the cortex showed a loss of embryo polarity, whereas genetic manipulations that maintain centrosome's close proximity to the cortex suppresses these phenotypes (Lyczak et al., 2006; Fortin et al., 2010) . This indicates that centrosome proximity to the cortex may be essential for the cue. However, a different hypothesis suggests that the centrosome proximity to the cortex is not absolutely essential; rather it has been proposed that the centrosome initially signals at a distance to start the process but that the centrosome closeness to the cortex reduces the time necessary for symmetry breaking after meiosis (Bienkowska & Cowan, 2012; Sumiyoshi & Sugimoto, 2012). This theory is based on evidence that discrete regions of the embryo cortex visibly undergo a symmetry breaking event that can be visualized by analyzing when cytoplasmic granule movement in directed fields as opposed to other methods of detecting symmetry breaking such as by monitoring myosin movements. These results indicate that a protein emanates from the centrosome to initiate symmetry breaking. Although the authors do not believe that microtubules emanating from the centrosome are required for centrosome movement towards the cortex (based on gamma-tubulin depletion), cytoplasmic microtubules do appear to be partially required. Furthermore, the authors find that centrosome proximity to the cortex

causes the polarity to occur more quickly and robustly, indicating that polarity initiation of the centrosome from a distance is inefficient (Bienkowska & Cowan, 2012).

1.3. Cortical Flow is the Primary Polarizing Force that Results from the Centrosome Cue.

Investigations of polarity initiation have revealed multiple pathways from which embryonic asymmetry can arise; in particular, there appears to be myosin dependent and PAR-2 dependent pathways. PAR-2 mediated asymmetry appears to be independent of cortical flow that depends on myosin (Zonies et al., 2010). However, for wild type polarity the cortical flow of the actomyosin cortex is always necessary whereas PAR-2 is dispensable (Munro et al., 2004; Beatty et al., 2010) . Initiation of the cortical flow depends on the centrosome-derived cue, but after the cue, it is believed that the cortical flow results largely as a result of physical forces of the extant actomyosin meshwork. Indeed causing a physical break in the actomyosin cortex by mechanical means is sufficient to cause a cortical flow similar to the flow that the centrosome initiates, indicating that the cue from the centrosome may in fact cause a local inactivation of actomyosin (Mayer et al., 2010). Several ways exist to observe the cortical flow. Cortical flow is most easily observed when cytoplasm and elements within the cytoplasm flow from the posterior into the anterior at the same time posterior cortical smoothing occurs (Hird and White, 1993; Munro et al., 2004). This displacement of cytoplasm at the cortex is accompanied by a consequent cytoplasmic streaming, which is when cytoplasm

elements flow through the center of the long axis of the embryo from the anterior to the posterior. Another way to observe cortical flow is to observe proteins. For example, examining myosin, or the anterior PARs is an efficient way to monitor this process since each of these proteins move from posterior to anterior (Munro et al., 2004; Motegi et al., 2006). Myosin in particular is useful for monitoring the flow because *C. elegans* embryos contain large and easy-to-observe NMY-2 foci which become asymmetric during establishment, and later transition into smaller structures that remain asymmetric. Also, NMY-2 along with RHO-1 and actin itself are critical components of the actin cytoskeleton that are required to generate the cortical contractile force. A loss of NMY-2, its regulatory light chain MLC-4, or the myosin chaperone UNC-45 will cause a loss of cortical contractility, and a resulting loss of cortical flow (Guo and Kemphues, 1996; Shelton et al., 1999; Kachur et al., 2008). Whenever there is a complete loss of cortical flow, strong asymmetries in the PARs and cytoplasmic cell fate determinants are lost. Therefore, the cortical flow in one-cell embryos is required for polarization and development .

1.4. The PAR proteins Contribute to Polarity Establishment.

Both the anterior PARs and PAR-2 contribute to the polarity establishment process. The anterior PARs contribute by positively promoting cortical flow (Cheeks et al., 2004; Munro et al., 2004). During the actomyosin flow, PAR-3, PAR-6, and PKC-3 clear from the posterior. During this clearing it has been shown that PAR-6 moves along with the actomyosin network (Munro et al., 2004). PAR-3, PAR-6, and PKC-3 are not passive hitchhikers during this process. Instead it seems the

anterior PARs positively regulate the extent to which cortical flow occurs. In the absence of PAR-3 myosin does not clear as far from the posterior nor does an asymmetric actin cap form (Kirby et al., 1990; Munro et al., 2004). Therefore, the activities of the anterior PAR proteins promote establishment even though establishment proceeds to a degree in their absence.

PAR-2 has its own role in initiating polarity in a way that may be independent of actomyosin function (Motegi et al., 2011; Zonies et al., 2010). Embryos often have no detectable polarity when centrosome assembly is compromised (Hamill, Severson, Carter, & Bowerman, 2002; O'Connell et al., 2000), and the anterior PARs, PAR-3, PAR-6, and PKC-3 persist over the whole cortex whereas PAR-2 can be seen at both poles or weakly all over the cortex. However, when actomyosin establishment is impaired in ways that do not interfere with centrosome function, asymmetries still arise (Zonies et al., 2010). This asymmetry depends on PAR-2 and the microtubules that emanate from the centrosomes (Motegi et al., 2011). Although PAR-2 has no clear homologs outside of nematodes it is essential in *C. elegans* one-cell embryos because of its role in exclusion of the anterior PAR proteins at the maintenance phase (see below). It has now been found that PAR-2 binds to microtubules in the posterior near the centrosome, and that by doing so initiates an auxiliary pathway in polarity that can cause anterior-posterior PAR asymmetries in a way that is independent of cortical flow. Therefore the PARs actively contribute to polarity establishment in addition to their prominent roles in polarity maintenance.

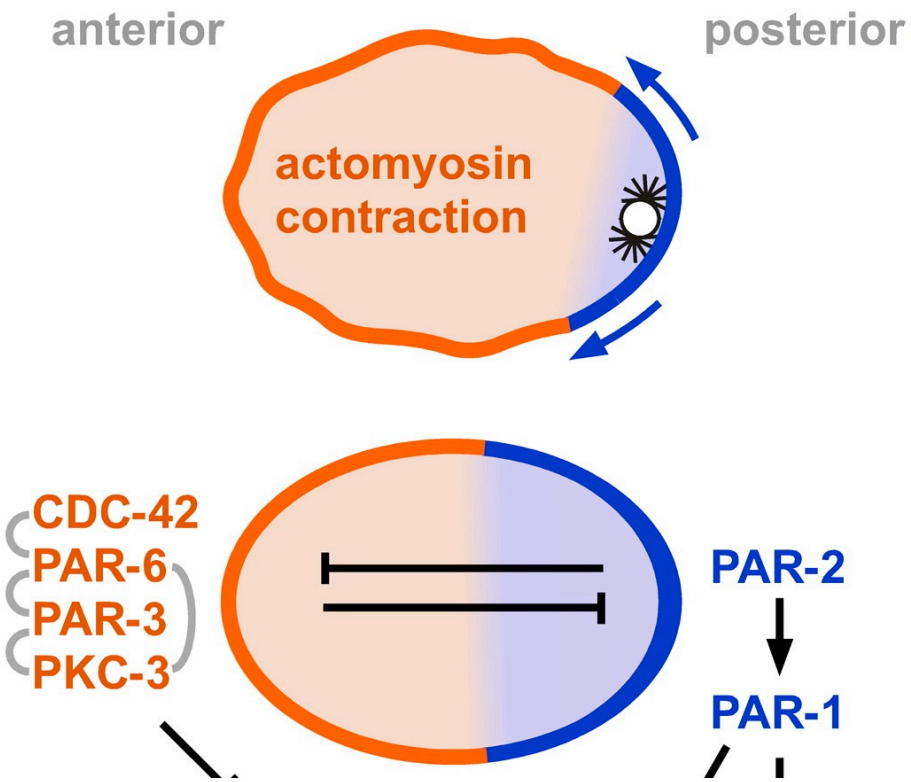
1.5. The Polarity Maintenance Phase of the One-cell Embryo.

After polarity is established in the early embryo, the PAR proteins maintain distinct anterior and posterior domains through the first cell cycle. The primary mechanism for this is based on a model that the anterior and posterior domains mutually exclude one another. A secondary mechanism that also appears to be necessary is that the PARs positively feedback their own localization by recruiting more PAR proteins to the cortex. Lastly, the PARs influence the function of the actomyosin cytoskeleton and possibly microtubules to further propagate asymmetry in the one-cell embryo.

PAR-6, PAR-3, PKC-3, and the small GTPase CDC-42 have been shown physically interact and localize to the anterior in an inter-dependent fashion (Etemad-Moghadam et al., 1995; Tabuse et al., 1998; Hung and Kemphues, 1999; Aceto et al., 2006; Li, Kim, et al., 2010) . All four of these proteins are co-dependent for their localization at the cortex. Furthermore, they act together to exclude the posterior PARs from the anterior (Figure 1.3). PKC-3 is believed to be the primary biochemical workhorse that uses phosphorylation to exclude posterior PARs from the anterior. PKC-3 can physically interact with both PAR-3 and PAR-6, which promote its localization and stability at the cortex (Tabuse et al., 1998; Li et al., 2010; Beers and Kemphues, 2006). Since both PAR-3 and PAR-6 both contain PDZ protein-protein interaction domains (found in PSD-95, Discs large, ZO-1) they are presumed to act in part as scaffolding proteins. For PAR-3 there is good evidence that the second of its three PDZ domains is required for its function although PAR-3 still functions when the first or third PDZ are (Li et al., 2010). The PAR-6 PDZ

domain also physically interacts with PAR-3, most likely by the first PDZ in PAR-3, but this interaction is not required for the co-localization or function of these proteins (Li, Kim, et al., 2010). Another type of scaffolding interaction is that PAR-3 requires oligomerization with other PAR-3 molecules to function (Li et al., 2010; Dawes and Munro, 2011). Finally, CDC-42 has been found to be required for polarity maintenance and is also asymmetrically enriched in the anterior at the maintenance phase (Kay and Hunter, 2001; Gotta et al., 2001; Schonegg and Hyman, 2006; Aceto et al., 2006; Kumfer et al., 2010). CDC-42, like the anterior PARs contributes to the robustness of cortical flow during polarity establishment (Motegi & Sugimoto, 2006; Schonegg & Hyman, 2006). CDC-42 is required for the localization of PAR-3 and PAR-6 (Kay and Hunter, 2001; M Gotta et al., 2001; Schonegg and Hyman, 2006), and the physical association of CDC-42 and PAR-6 is required for its activities (Aceto et al., 2006), indicating that CDC-42 and the function of the other anterior PARs are inter-dependent as well. Thus, anterior PARs and their functions are crucial to maintain embryo asymmetry and function. They achieve this by down-regulating the posterior, promoting self and other anterior PAR functions, and by acting as a control point for cytoskeletal regulation.

Figure 1.3.



Anterior and posterior PAR localization during the establishment and maintenance phases. During establishment the anterior PARs (orange) leave the posterior, a process dependent on the centrosome derived signal. During this time, the absence of the anterior PARs allows posterior PARs (blue) to become recruited and enriched to the posterior cortex. After two distinct domains are formed, the PAR groups mutually exclude one another from localization of their respective cortical domains. This figure has been modified from (Bob Goldstein & Macara, 2007) and used with permission for this thesis only, and should not be cited directly as the original work of this thesis.

The proteins that act to maintain polarity in the posterior appear to function by similar mechanisms as the anterior PARs. For example PAR-2 localizes to the posterior cortex (Boyd et al., 1996) and may bind and recruit PAR-1 to the posterior based on evidence that they physically interact (Motegi et al., 2011). PAR-2 may localize to the cortex in two ways one of which is by binding microtubules, and the second of which may be by binding membranes (Motegi et al., 2011). Intriguingly, in addition to PAR-2's proposed ability to recruit PAR-1 to the cortex, it also may permissively recruit more PAR-2 protein to the cortex after PAR-2 domains are established (Motegi et al., 2011). One result that supports this idea is that microtubule binding deficient PAR-2::GFP is able to be recruited to the posterior if WT PAR-2 is present to establish a PAR-2 enriched region of the cortex, even when there is no cortical flow. For these reasons it appears that PAR-2 may promote self and PAR-1 localization as a mechanism to both establish and maintain a posterior domain. The second mechanism the PARs use to maintain asymmetry is by mutually excluding one another from either the anterior or posterior cortex. PKC-3 is believed to be responsible for phosphorylating and down-regulating PAR-2 and LGL-1 (Hao et al., 2006; Hoege et al., 2010; Beatty et al., 2010). There is also evidence that there is a potent mechanism by which the posterior PARs exclude anterior ones. PAR-1 and PAR-4 are kinases that may play a role in excluding the anterior PARs from the posterior (Hung and Kemphues, 1999; Motegi et al., 2011). In the case of PAR-1, PAR-3 may be a direct target (Motegi et al., 2011), although this activity may be masked by redundancy, since *par-1* mutants have no effect on the

size of the anterior domain (Etemad-Moghadam et al, 1995). Less is understood about the function of the cytoplasmic and uniformly cortical PAR-4 (Watts, Morton, Bestman, & Kemphues, 2000). *par-4* mutants have larger anterior domain sizes indicating it may act in part to restrict anterior PARs or somehow support the posterior PARs. In mammals the PAR-4 homolog LKB1 is an AMP kinase regulator that is able to activate homologs of PAR-1 by phosphorylating them on their T-loops (reviewed in Alessi et al., 2006). PAR-1 homologs such as MARK2 in turn influence microtubule organization in epithelial cells and neurons (reviewed in Matenia and Mandelkow, 2009). PAR-2 and LGL-1 also localize to the posterior, are not kinases, and are not thought to be scaffolds. However both proteins appear to have the ability to exclude anterior PARs from the posterior (Beatty et al., 2010). PAR-2 contains a catalytic RING (Really Interesting New Gene) domain that is a likely E3 ubiquitin ligase (Levitan et al., 1994). The RING catalytic activity is necessary for full PAR-2 activity (Levitan et al., 1994; Hao et al., 2006), but nothing is known about what substrates could be ubiquitylated by PAR-2. Similarly, the widely conserved LGL-1 protein has functions that are not well defined; it has been proposed to either directly act through myosin regulation or as an antagonist to anterior proteins such as PAR-3 or PKC-3 (Peng et al., 2000; Wirtz-Peitz et al., 2008; Beatty et al., 2010; Hoege et al., 2010). Finally, PAR-5 is a 14-3-3 protein that is required for the anterior and posterior PARs to exclude one another from their respective cortical domains (Morton et al., 2002), and is thought to function by binding and removing phosphorylated proteins from the cortex. This important function is believed to be necessary to remove both anterior and posterior proteins which are

phosphorylated; as a result, the loss of PAR-5 causes overlap of the anterior and posterior PAR markers. Thus, localization of PARs to the cortex is dependent on positive feedback to promote protein localization at the cortex, and then mutual exclusion of proteins from the posterior or anterior cortex to maintain asymmetry.

Overall, much has been learned about how the PARs and their homologs maintain the anterior-posterior as well as apical-basal domains in cells. However, the role of some polarity proteins, such as PAR-2 and LGL-1 are only beginning to be understood. LGL-1, a protein also conserved in vertebrates, was recently found to be able to be overexpressed to compensate for the loss of PAR-2 (Hoege et al., 2010; Beatty et al., 2010). LGL-1 plays a role in maintaining *C. elegans* posterior polarity, but it is not known how it functions whereas it is believed that PAR-2 may act in the ubiquitin pathway (Levitan et al., 1994; Boyd et al., 1996). The requirement for PAR-2 highlights the notion that ubiquitylation may regulate the PARs.

Part Two. The Role of Ubiquitin Pathway Proteins in Polarizing the *C. elegans* Early Embryo.

The process of polarization in *C. elegans* one-cell embryos is extremely well characterized at functional and genetic levels. However, much more remains to be learned concerning the specific biochemical modifications and regulatory events that control this process. For polarity, phosphorylation plays a prominent and relatively well-understood role. However, the specific regulatory events that ubiquitin mediates are largely unknown. Here I provide a review of what is known concerning how ubiquitin regulates the early events in polarity.

It is likely that many events in the early embryo such as the cell cycle and polarity are regulated by ubiquitylation and protein degradation. Examples of genes involved in ubiquitylation and/or protein degradation pathways include conserved ubiquitylation ligation complexes such as the cullins, E3 ubiquitin ligases, and even the proteasome (Liu et al., 2004; Kipreos, 2005; Moore and Lynn Boyd, 2004; Sugiyama et al., 2008). There are two main categories of ubiquitin-regulatory genes that are known to influence embryonic polarity. One set is comprised of essential genes such as PAR-2, CUL-2, RPN-1, RPN-2, and ZYG-11, which have been reported to cause lethality when mutated or depleted (Levitan et al., 1994; Liu et al., 2004; Sugiyama et al., 2008). The second set consists of genes that have been reported to have non-lethal phenotypes that influence the polarity process such as the *ceBRATs,* FEM-3, and RPN-10 (Labbé et al., 2006; Pacquelet et al., 2008; Hyenne et al., 2008). For each of these genes, there is a known or predicted interaction with ubiquitin dependent pathways. A varying amount of information is known about how each of these genes function. One underlying theme is that the genes have distinct functions in separate pathways suggesting that there could be five or more different kinds of ubiquitin based regulatory events that impinge upon polarity. In every case clearly delineated biochemical targets are not known.

2.1. The Highly Conserved CUL-2 Complex and its Roles in Embryonic Polarity.

Cullins are conserved scaffolding proteins that recruit multiple proteins including E3 ubiquitin ligase subunits and substrate adapters to form ubiquitin ligation complexes. The ability of cullin complexes to have different substrate

recognition components (SRCs) means that an individual cullin complex can be involved in ubiquitylating a variety of substrates. For example, in *C. elegans,* the cullin CUL-2 and the SRC ZYG-11 are partially required to regulate cyclin B1 levels (Liu et al., 2004; Sonneville and Pierre Gönczy, 2004; Vasudevan et al., 2007). Failure of cyclin B1 degradation causes an extension of meiosis II. As a result the persistent meiotic spindle can initiate a microtubule-dependent reversal of polarity or the formation of ectopic PAR-2 domains. Thus the effect of *zyg-11* and *cul-2* on polarity can be attributed in part to meiotic delay. However, *cul-2,* but not *zyg-11* has an additional function that involves restricting PAR-2 from the cortex. *cul-2* and *tubulin tbb-2* double RNAi results in the formation of ectopic PAR-2 domains at the cortex, whereas *tbb-2* depletion results in embryos with no PAR-2 domains (Liu et al., 2004). This indicates that CUL-2 may function to prevent PAR-2 accumulation at the cortex when microtubules are removed from the embryo. A caveat with this interpretation from Liu *et al,* 2004 is that a double RNAi of *cul-2* and *tbb-2* may not deplete microtubules as strongly as a *tbb-2(RNAi);* thereby creating a situation in which microtubules from the centrosome can still recruit PAR-2. In summary, it is plausible that CUL-2 has the ability to block PAR-2 cortical accumulation and it is certainly the case that both CUL-2 and ZYG-11 act to promote the completion of meiosis, which removes the meiotic spindle as a trigger for ectopic PAR-2 recruitment.

A second specific role of CUL-2 is to act with the SRCs FEM-1/2/3 components to influence PAR-6 protein levels. In a screen for *par-2(it5ts)* suppressors *nos-3,* a homolog of Drosophila *nanos,* was found to be able to potently

suppress *par-2(it5ts)* embryonic lethality at semi-permissive temperatures (Labbé et al., 2006). One of the known roles of NOS-3 is that it participates in a complex with FBF-1 to post-transcriptionally repress FEM-3 translation (Kraemer et al., 1999). Once translated, FEM-3 acts as an SRC with the ubiquitin ligase CUL-2 (Starostina et al., 2007). Thus Paquelet *et al.* found that depletion or mutation of FEM-3 and its homologs FEM-1 and FEM-2 can suppress *par-2(it5ts)* phenotypes. Then, PAR-6 protein levels were found to be reduced upon inhibition of the *fem* genes or *cul-2.* Furthermore, in immunoprecipitations, PAR-6 physically interacts with FEM-3. Thus there is strong evidence that CUL-2 along with the FEM SRCs act to repress PAR-6 levels, possibly through ubiquitylation. When considered as a whole, these studies indicate that CUL-2 may have a role as a negative regulator of both anterior (PAR-6) and posterior (PAR-2) protein functions in one-cell embryos.

2.2. The Crucial Polarity Regulators PAR-2 and PAR-1.

The putative ubiquitin ligase PAR-2 is a crucial component of the *C. elegans* embryonic polarity pathway. Much is known about how the gene functions, but it is not known whether PAR-2 is a genuine ubiquitin ligase and what PAR-2's substrates may be. PAR-2 was cloned and characterized as a RING zinc finger and ATPase domain containing protein (Figure 1.4) (Levitan et al., 1994). PAR-2 also localizes to the posterior cortex in one-cell embryos and is required to exclude anterior PAR proteins from entering the posterior (Boyd et al., 1996; Watts et al., 1996). Several functions of PAR-2 have been revealed by mutational analysis. One important result is that mutation of PAR-2's RING domain destabilizes PAR-2 at the cortex, and thus

the RING domain is required for PAR-2's ability to mutually exclude the anterior PARs during the maintenance phase (Hao et al., 2006). To date, the importance of PAR-2's RING finger domain for its function is the only existing evidence that PAR-2 may be an enzymatically active ubiquitin ligase. A related result is that when PAR-2's RING finger is mutated, it may cause PAR-2 accumulation in embryos, indicating that PAR-2 could self-ubiquitylate. Thus one of strongest and most sensible hypotheses about how PAR-2 functions is that it may ubiquitylate anterior PAR proteins to negatively regulate their function or stability at the cortex, and possibly control its own stability.

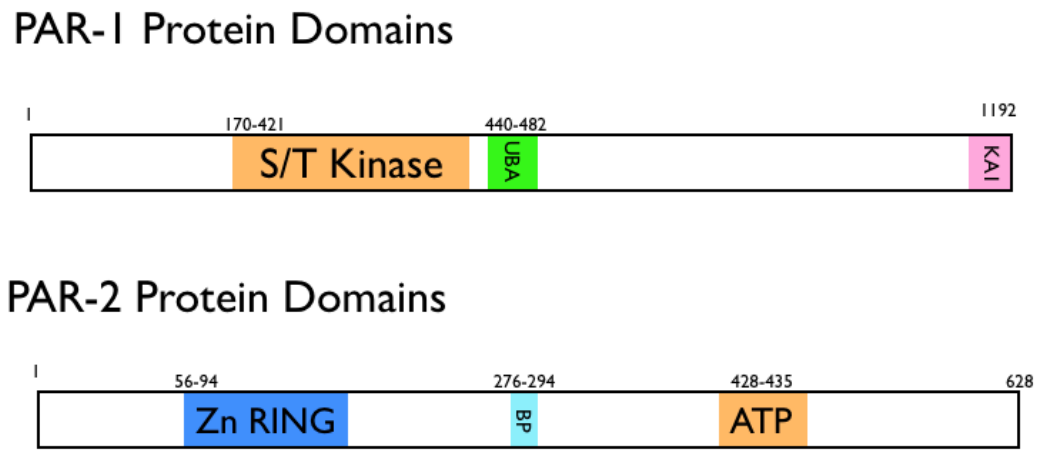
In addition to its possible role as an enzyme, some results indicate that PAR-2 may also act as a molecular tether to cytoskeletal, membrane, and protein elements in the early embryo. By expressing small parts of PAR-2 it was found that a region in the center of the protein is required for PAR-2 to localize to the cortex of early embryos and that PKC-3 phosphorylation de-stabilizes PAR-2 at the cortex (Hao et al., 2006). It was later found that PAR-2 also has the ability to bind microtubules, phospholipids, and to complex with the PAR-1 protein (Motegi et al., 2011). There is a complex interplay of these PAR-2 functions. For example, microtubule binding appears to protect PAR-2 from PKC-3 phosphorylation, and the absence of PKC-3 phosphorylation appears to allow PAR-2 to bind phospholipids. The evidence suggesting that PAR-2 may complex with PAR-1 suggests that PAR-2 may also act to recruit PAR-1 at polarity establishment or to stabilize it during polarity maintenance.

PAR-1 is a serine-threonine kinase that has homology to the mammalian microtubule affinity-regulating kinases (MARKs) (Figure 1.4). PAR-1 protein is cortically localized to the posterior of the embryo, and is required for the asymmetry of cell fate determinants such as P granules, SKN-1, MEX-5 and others (Kemphues et al., 1988; Guo and Kemphues, 1995; Tenlen et al., 2008). There is good evidence that PAR-1 acts by phosphorylating proteins such as MEX-5 (Tenlen et al., 2008). It is also believed that PAR-1 may phosphorylate PAR-3 to exclude PAR-3 from the posterior (Motegi et al., 2011). In addition to a conserved kinase domain, PAR-1 also contains a ubiquitin associated UBA domain which is adjacent to the C-terminal end of the kinase domain. Typically, UBA domains bind to poly-ubiquitin chains or to other beta-grasp like proteins (Wilkinson et al., 2001). It is not known whether the UBA domain of PAR-1 is important for its function, but it has been noted that PAR-1::GFP with a deleted UBA domain does not function normally (Geraldine Seydoux, personal communication cited by Jin Li, Ph.D. dissertation). Thus, PAR-1 may interact with poly-ubiquitylated proteins in *C. elegans.*

The mammalian MARK proteins are generally considered to be the closest homologs of *C. elegans* PAR-1. The MARKs are known for their ability to phosphorylate microtubule-associated proteins (MAPs), thereby regulating the affinity of those proteins for microtubules (Drewes et al., 1998), and have been highly studied because of their functions in neurons and other cell types (Trinczek et al., 2004). MARK regulation can affect the function of the microtubule cytoskeleton; for example, MARK-4 either directly or indirectly through a MAP localizes to the centrosome and can influence microtubule morphology when

mutated or overexpressed (Trinczek et al., 2004). In one proteomic study of mammalian PARs and polarity effectors MARK4 was found to co-immunoprecipitate with two ubiquitin-related proteins, USP7 and USP9 (Brajenovic et al., 2004). A follow-up study of this observation led to the discovery that without the DUB USP9X, ubiquitylation of MARK4 is increased and activation of MARK4 by phosphorylation at its T-loop is decreased. Their overall results imply that UBA domain association with a ubiquitin chain on PAR-1 itself may regulate the kinase activity. Crystal structures have shown that the UBA domain in MARK4 also associates with the MARK4 kinase domain. Three studies have observed the structural relationship between the UBA domain and kinase activity, and have attempted to determine whether the UBA-kinase domain association is common in active or inactive MARK4 (Jaleel et al., 2006; Marx et al., 2006; Murphy et al., 2007). There is still some controversy in the field about which is the case but more evidence suggests that UBA-kinase association is common in activated MARK4 (Marx et al., 2006; Murphy et al., 2007). What this suggests for *C. elegans* is that the UBA domain containing PAR-1 may be ubiquitylated and de-ubiquitylated as a regulatory switch although this hypothesis has not been tested.

Figure 1.4.



The domain architecture of PAR-1 and PAR-2. Shown are the protein models of PAR-1 and PAR-2 oriented from the N terminus to C terminus. The amino acids at which domains begin and end are labeled. Abbreviations stand for serine-threonine kinase domain, ubiquitin associated domain (UBA), kinase associated domain 1 (KA1), zinc RING finger domain, and ATPase homology domain (ATP). BP is a basic patch of PAR-2 amino acids that is closely conserved between other *Caenorhabditis* species.

2.3. The *C. elegans* Homologs of *D. melanogaster* BRAT are Potential Ubiquitin Ligases that Suppress *par-2* Loss of Function.

Homologs of the *D. melanogaster* BRAT proteins are suppressors of *par-2(ts)* when either mutated or depleted by RNAi (Labbé et al., 2006; Hyenne et al., 2008). In fruit flies, the *brat* gene functions in the same pathway as *nanos.* Therefore the *C. elegans* BRAT homologs might be suspected to suppress *par-2(ts)* phenotypes *via* the *nos-3* pathway that regulates PAR-6 levels (A Pacquelet et al., 2008). However Hyenne and colleagues found that PAR-6 levels are not changed when any of four different *brat* homologs (*ncl-1, nhl-1, nhl-2, and nhl-3)* are mutated. Amongst these four genes, with *ncl-1* and *nhl-2* are the strongest suppressors of *par-2(ts)*. Since all four *brat* homologs suppress *par-2* mutations without affecting PAR-6 levels, the *brat* homologs are likely affect embryonic polarity *via* a pathway independent of *nanos* (Hyenne, Desrosiers, & Labbé, 2008). This suppression results in the restoration of mutant *par-2(ts)* protein to the posterior cortex, suggesting that the suppression restores PAR-2 function instead of bypassing it. Finally, mutation of the *brats* alone causes extremely slight and non-lethal defects in polarity such as more synchrony at the second cleavage (when AB and P1 divide). Thus the *brats* have some definite role in polarity, which is weak and also masked by a high level of genetic redundancy. The mechanism by which they perform this suppression is unknown, but two hypotheses exist. One possibility suggested by studies of the *D. melanogaster* BRAT protein is that the *C. elegans* homologs function as translational inhibitors (Sonoda and Wharton, 2001; Betschinger et al., 2006). A second

possibility suggested by the observation that the RING domain is present in four of five *brat* homologs in *C. elegans* is that the proteins may participate in ubiquitin ligation (Hyenne et al., 2008). However further research on the most potent *brat* suppressor of *par-2, nhl-2* has suggested a third possible mechanism. Hammell and colleagues found that NHL-2 participates in a complex of proteins called miRISC, which is a micro-RNA silencing complex (Hammell et al., 2009). Therefore, *nhl-2* may down-regulate unknown mRNAs that in turn affect polarity in the early embryo. Of course, the role of NHL-2 as a miRISC component does not exclude a parallel role as a translational repressor or ubiquitin ligase.

2.4. The Role of the Proteasome in the early embryo

The 26S proteasome is a highly conserved eukaryotic complex that is known for its role in recognizing and degrading ubiquitylated proteins (Raiborg & Stenmark, 2009; Ventii & Wilkinson, 2008). *C. elegans* contains homologs to most of the well-studied proteasomal proteins, and many of these physically interact, supporting the idea that *C. elegans* has a working proteasome (Hershko & Ciechanover, 1992; Ventii & Wilkinson, 2008). Two studies have found early embryonic roles for the proteasome proteins *rpn-2* and *rpn-10* (Labbé et al., 2006; Sugiyama et al., 2008).

RPN-2 is an essential regulatory subunit of the proteasome which, when depleted, leads to high embryonic lethality. Furthermore, as a subunit of the proteasome it is likely to be involved in a myriad of cellular processes. Therefore the gene was not studied closely in the context of the early embryo until Sugiyama

and colleagues discovered in a yeast two-hybrid screen that RPN-2 physically interacts with PKC-3, and confirmed this physical interaction in cell culture extracts. They then found that RPN-2, and another subunit, RPN-1 when depleted recapitulated mitotic spindle orientation defects common in anterior PAR mutants in AB at the two cell stage. Specifically, they found that the nuclear-centrosome complex rotated in AB similar to what normally occurs in the P1 cell leading to a longitudinal mitotic axis in AB instead of a transverse axis. Interestingly, when the authors closely examined cell size asymmetry and polarity markers (such as PKC-3 and PAR-1), polarity appeared to be completely normal. This was unique because traditionally a mitotic spindle rotation in AB was usually observed when the anterior PARs were defective and when cell polarity was disrupted (Etemad-Moghadam et al., 1995). Although these data do not reveal the nature of the relationship between PKC-3 and the proteasome directly, the fact that mitotic spindle rotation is downstream of anterior PAR function and localization suggests that the proteasome's role in this process is also downstream of the PARs. The data also support that proteasome dysfunction uncouples mitotic spindle rotation in AB from the function of the anterior PARs.

RPN-10 is a homolog of a baker's yeast protein by the same name, and is generally thought to be a proteasomal receptor protein that binds and increases the affinity of ubiquitylated proteins for the proteasome (Mayor et al., 2005). In *C. elegans* the gene is less essential than genes encoding components of the proteasome, and when mutated does not lead to lethality, although it can cause sterility (Shimada et al., 2006). Furthermore, the studied phenotypes of RPN-10

support the notion that it is a proteasomal receptor that affects the degradation of specific proteins as well as general protein ubiquitylation levels (Shimada et al., 2006). In 2006 Labbé and colleagues found that *rpn-10* depletion or mutation can suppress *par-2(it5ts)* and *par-2(RNAi)* defects (Labbé et al., 2006). Thus, RPN-10 is involved in polarity at the one-cell stage, and acts by either negatively regulating the function of posterior PARs or by promoting the function of anterior PARs. These results are also consistent with the findings that RPN-2 has functions in the AB cell, suggesting that the proteasome generally functions to promote anterior PAR function. However, it is not clear whether the effects of *rpn-10* mutation are the same as proteasomal genes *rpn-1* and *rpn-2.* The evidence that *rpn-10* can suppress polarity defects at the one-cell stage indicates that its targets may be important for early polarity. In contrast, *rpn-1* and *rpn-2* are non-essential for one-cell embryo polarity but are necessary at the two-cell stage. Overall though it is clear that the proteasome has active roles early in embryogenesis.

2.5. The Anaphase Promoting Complex and the POD Mutations.

One final ubiquitylation machine that plays a role in embryonic polarity is the anaphase promoting complex (APC) (Rappleye et al., 2002). Mutation or depletion of the APC can have pleiotropic defects during development, but hypomorphic mutations that presumably only partially impair the APC can lead to polarity defects. Polarity and osmosis defective mutants or PODs (*pod-3/4/5/6*) constitute one such class of APC mutations. In addition to polarity defects, *pod* mutant one-cell embryos are also osmotically defective. PAR-2 does not localize normally, but

accumulates in cytoplasmic vesicles. By investigating known APC pathways, Rappleye and colleagues (Rappleye et al., 2002) found that the APC *pods* appear to act through a pathway that terminates in *securin* activation. Securin depletion matched the phenotypes of *pod* mutations, and furthermore, the authors went on to hypothesize that securin promotes association of the Sperm-Pronucleus Centrosome Complex (SPCC) with the cortex. Thus, although it is not clear, it is possible that the APC contributes to polarization by either promoting establishment *via* centrosome association with the cortex, or by maintaining PAR-2 at the cortex, or both.

Part Three. Polarity Establishment In Yeast.

3.1. Fission Yeast

Cell polarization has also been studied in the fission yeast *Schizosaccharomyces pombe* (Raiborg & Stenmark, 2009; Ventii & Wilkinson, 2008). During interphase *S. pombe* cells grow in a roughly cylindrical shape and when fission occurs, they undergo cytokinesis creating two roughly equally sized descendants. Unlike *C. elegans,* yeast lack PAR proteins and so some aspects of regulating polarity of a yeast cell must differ from *C. elegans*, but other aspects may be common to both organisms.

At the time of division, fission yeast are bi-symmetric with two indistinguishable cortical growth ends that are different from the lateral sides of the cell. However, after division daughter cells are asymmetric because one end of the cell was previously the "old growth end" where active Cdc42 and the Tea1-Tea4

complex would have been localized. Whereas the pole at which fission occurred must be newly defined as a "new growth end". At G2 the non-growth pole of a fission yeast is defined, and polarity factors localize to the new site. This process is often called new end take off or NETO (reviewed in Martin and Chang, 2005). During interphase, both poles of the cell will have cortically enriched Tea1-Tea4 complex as well as the DYRK family kinase Pom1 (Mata and Nurse, 1997; Martin et al., 2005). At the beginning of mitosis, as in other organisms, Cdc42 becomes activated at the new growth end and the old growth end of the cell. The cortically enriched kinase Pom1 allows for Cdc42 activation by excluding the Cdc42 inactivating GAP Rga4 from the poles (Tatebe et al., 2008). NETO requires the Tea1-Tea4 complex which causes many downstream effects that include recruiting the formin For3 by Tea4 which later promotes polarized actin cables in the cell (Martin et al., 2005). In addition to For3, cell wall modifying enzymes are recruited and active Cdc42 becomes enriched. Therefore the Tea1-Tea4 complex is an important tool for fission yeast to define a new pole; a process required for further growth and cell divisions.

The Tea1-Tea4 complex and microtubules play a prominent role in defining the cell growth poles in *S. pombe.* In the absence of either microtubules, Tea1, or Tea4 cell tips can ectopically form on the side of cells causing fission yeast to branch into a T shape instead of cylindrically. In the case of the Tea1-Tea4 complex this makes sense because it is required to define new growth ends, and without it new growth begin at unusual locations at the yeast cortex. One reason microtubules are required is that they are necessary for Tea1 to be deposited and enriched at the

cortex. Tea1 can be visualized prominently on + ends of microtubules and is known to complex with Tea2 and Mal3. Tea2 is a kinesin which transports Tea1 to microtubule + ends and Mal3 is microtubule binding protein that may stabilize microtubules and possibly recruits Tea2 (H Browning et al., 2000; Heidi Browning and Hackney, 2005). Thus, the localization of the Tea1-Tea4 complex depends on the localization of microtubules ends to the appropriate part of the cortex. A protein called Tip1 plays an important role here because Tip1 is deposited at the cortex by microtubule tips, and in turn stabilizes microtubules at the cortex where it is enriched. Tip1 is partially responsible for defining where the Tea1-Tea4 complex will localize. Therefore the working model is that microtubule + ends extend from an MTOC near the nucleus of the cell towards the tips where Tip1 is deposited, and in turn the Tea1-Tea4 complex is deposited. It has been hypothesized that microtubule tips favor the ends of the cylindrical fission yeast cell shape, and that thus the cell shape itself promotes Tip1 deposition to the correct part of the cortex. Indeed if yeast cells are artificially grown in a way so that the cells are curved, the NETO can be defined at the area where microtubules can contact the cortex instead of at the end of the cylinder (Terenna et al., 2008). Finally, some markers like Bud6, Mal3, and Moe1 become enriched where microtubules contact the cortex, and their cortical enrichment does not depend on Tip1 (Chen et al., 2000; Minc et al., 2009). This indicates that microtubules define new ends and polarize cells by multiple pathways parallel to Tip1. Therefore microtubules play a prominent role in polarizing fission yeast cells. Some parts of this mechanism, such as the transport and role of Tea1 are well defined, whereas any parallel mechanisms are less defined.

In summary, yeast must establish new growth ends so that they behave identically to the old growth ends at mitosis. This is achieved in part by depositing polarity proteins at the cortex *via* the tips of microtubules. This is a relevant issue for *C. elegans* when considering whether microtubules can be part of an evolutionarily conserved polarity establishing pathway.

One of the reasons why I believe it is interesting to draw parallels between fission yeast and *C. elegans* polarity initiation is because de-ubiquitylating enzymes (DUBs) are also required for polarity in both organisms. Later in this thesis, I provide evidence about how DUBs are required in *C. elegans,* and in this section I will describe some of what is known for *S. pombe.* One study, Kouranti et al., 2010 explored the twenty DUBs present in the *S. pombe* genome (Kouranti et al., 2010). In their study they analyzed mutant phenotypes, DUB localizations, and also DUB complexes (*via* proteomic analysis of pulled down DUBs). The authors first examined the localization patterns of DUBs, and based on common localizations and common gene ancestry, were able to predict some DUBs that might work together. This was an important step since most DUBs have mild or no phenotypes when mutated; most of the phenotypes are slow growth phenotypes. Some DUBs that had common patterns, and that are important later in this thesis is the group Ubp4, Ubp5, Ubp9, Ubp15, and Sst2. This group of DUBs had localization patterns that suggested they might be important for cell polarity and endocytosis. Specifically Sst2, Ubp4, Ubp5, Ubp15, and Ubp9 were all found to localize close to the cell septum and to vesicles near the septum. In the case of Ubp4, it was confirmed to co-localize with vesicle marker Pan1, and endocytosis marker. Ubp5 and Ubp15 are

close homologs which both localize to cytoplasmic vesicles, and in addition Ubp15 localizes to the nucleus. Ubp5 co-localization with Vrg4 indicates that it localizes to the Golgi apparatus. Ubp9 localized to both the cell septum and also the cortex at the cell tips. Most, but not all other DUBs were found to localize either uniformly in the cytoplasm or in the nucleus. When the authors deleted four of these septum localizing DUBs (Ubp4, Sst2, Ubp5, and Ubp15) the *S. pombe* line was surprisingly viable. However, by mutating the final DUB that localized to the septum and to the cell cortex, Ubp9, the cells showed severe phenotypes. The quintuple deletion was inviable, and was partially defective in the uptake FM4-64 dye into endocytic vesicles. Normally endocytosis is more active at the cell tips or growth ends of the *S. pombe* cell. However in the quintuple mutant, FM4-64 dye uptake was reduced, indicating a potential failure in endocytosis, and FM4-64 vesicles that did form were more likely to be on the lateral sides of the cell indicating that the endocytosis preference that exists in a polarized cell was absent. Two cell membrane proteins, Can1 and Pub1 were shown to have higher ubiquitylation levels in the absence of these five DUBs indicating that they may be potential targets for deubiquitylation. The authors also found evidence that some DUBs work in protein complexes that control DUB localization. In particular they found that Ubp15 and the protein Ftp105 co-localize on cellular structures, but that UBP15 localization relies on Ftp105; deletions of *ftp105* led to a failure of Ubp15 to localize, but not vice versa. The protein Ubp9 was found to complex with Bun107 and Bun62. Through mutation it was found that Bun107 restricts Ubp9 and Bun62 from the nucleus and allows them to localize to the cortex. Bun107 and Bun62 also both localize to the

cortex in a way that depends on Ubp9. Overall, the results from this study suggest that a group of five DUBs with similar subcellular localization are necessary for endocytosis, and for endocytosis to occur in the normal location at cell tips. This suggests that the loss of these DUBs may cause the cells to lose polarity. Although to confirm this future studies should examine other polarity markers such as Tip1 and other related tip localizing proteins.

3.2. Baker's Yeast

Budding yeast determine a bud location based on previous septation axis. In particular, haploid yeast tend to form buds that are adjacent to the previous division site. Diploid daughters bud at a site 180° from their birth scar, referred to as a distal site, and yeast that have budded at least once choose a site near the site of previous budding, but tend to choose a distal site as they age (Chant and Pringle, 1995, reviewed in Roemer et al., 1996; Mata and Nurse, 1998). Mother budding yeast cells usually have bud scars which, for a while act as a landmark or tag. A ring of septins from cytokinesis (Cdc3, Cdc10, Cdc11, Cdc12) remains near the cortex in yeast and the septins are thought to be required for the formation of a nearby landmark that will serve as the future bud site. In fact, mutations of any of these four septins can result in a bipolar budding pattern indicating that their activity is required for bud site selection (Flescher et al., 1993). Other genes that influence bud site selection in haploid yeast are *AXL2/BUD10, BUD3, and BUD4*. These three proteins are known to localize to the bud neck in the G2 cell cycle phase. Bud3 is important for this process because it forms a double ringed structure at the bud neck similar to various septins that also remain as a cortical ring on both the mother and daughter

cell, marking the site of division. In both a and alpha cells, BUD3 protein determines a preference for the next axial bud site to be near the BUD3 landmark (Chant et al., 1995). After the actions of *AXL2/BUD10, BUD3, and BUD4* the proteins Cdc24, Cdc42, and Bem1 are required to polarize the yeast cell. Cdc42 becomes enriched at the bud site. The localization of the septins and other downstream proteins at the future bud site indicate that their activity likely promotes polarization and cell budding at those locations.

Since the septin ring seems to serve as the landmark between cell cycles, it appears to maintain a memory of how the yeast cell should divide. However, the septin ring landmark is not permanent, and when yeast starve for a period, the "memory" of the bud site landmark is lost. One way that this can be observed is that Cdc10 and Cdc11 leave the cortical septin ring complex during starvation (Pablo-Hernando et al., 2008). Then, there is evidence that the budding site preference is lost. At subsequent re-feeding, a yeast cell can bud either proximally or distally indicating that it does not prefer the proximal pole; the effect is much stronger in haploid yeast than diploid yeast. This indicates that the cortical landmarks' ability to define the bud site is temporary, and if erased, a bud site can be established de-novo. However, since haploid yeast become bipolar, this indicates that cells still retain the memory of the two proximal/distal poles. Because the starvation effect also preferentially affects haploid yeast it also may depend specifically on the actions of *AXL2/BUD10, BUD3, and BUD4.* Much less is known about how a new bud site is chosen when a cell grows from a spore.

Microtubule function is not essential for polarized growth, but the actin cytoskeleton and secretion activity are required (Pruyne and Bretscher, 2000). Bud site determination depends partly on septins and downstream regulators, whereas the bud site growth is initiated by Cdc24, Cdc42, and Bem1. Like yeast, asymmetry in *C. elegans* requires actin dependent processes and CDC-42 to maintain polarity (Hill and Strome, 1990; Gotta et al., 2001; Aceto et al., 2006). However, to establish polarity *C. elegans* requires the centrosome or astral microtubules (Tsai and Ahringer, 2007). Bud site determination is perhaps the closest parallel in yeast to the centrosome based symmetry breaking events in *C. elegans*. Could microtubules or the MTOC also break symmetry in baker's yeast? Unfortunately there is little evidence for such a hypothesis. This is primarily because initiation of the bud site, and polarization occurs in the presence of nocodazole, a microtubule de-polymerizing agent (Jacobs et al., 1988). However, it is clear that the bud site may still subtly influence the MTOC or vice versa. For example, the MTOC tends to be oriented to the side of the nucleus so that it faces the future bud site, and this perhaps indicates that the future bud site interacts with the microtubule cytoskeleton (Kilmartin and Adams, 1984; Jacobs et al., 1988). Furthermore, upon starvation and throughout sporulation, the septins Cdc10 and Cdc11 (genes also required for bud site determination) localize to spindle microtubules, and microtubules at the MTOC in the nucleus (Pablo-Hernando et al., 2008). Therefore, there is at least one connection between septin and microtubule function that isn't well understood. The starvation situation is important because this is also when haploid yeast can lose their preference for a bud site. Currently, there has been no

investigation into whether the choice of a new bud site after starvation depends in any way on the activity of microtubules, MTOC, or the septins that can localize to microtubules. Therefore, it is currently believed that microtubules play a minimal role in establishing polarity for *S. cerevisiae,* but more connections between the microtubule function, and bud site determination may emerge as a result of future investigations.

Part Four. Introduction Closing.

This introduction has reviewed and discussed polarization in *C. elegans* and what is known about ubiquitin-regulation of this process. During the next chapter of the thesis, I will present yet another pathway involving de-ubiquitylases (DUBs) that is required for polarity establishment. During this introduction I also reviewed polarity in two popular yeast models as well. DUBs are important for polarity in *Schizzosaccharomyces pombe.* All of these topics provide important context for the next chapter which examines the role of several DUBs in polarity of the *C. elegans* embryo. These DUBs are evolutionarily conserved and are also involved in polarity of *S. pombe.*

Chapter Two

This chapter has been modified from an original manuscript written by myself and edited with the help of Kenneth Kemphues and Diane Morton. I performed all experiments in this section.

Deubiquitylation Machinery is Required to Initiate Embryonic Polarity in *Caenorhabditis elegans*

Introduction

Eukaryotes, including *C. elegans*, regulate protein turnover through conserved ubiquitin-mediated degradation mechanisms (Kipreos, 2005). Ubiquitin conjugation requires the activity of E1, E2, and E3 enzymes, and results in the covalent attachment of mono-ubiquitin or poly-ubiquitin to a target protein (Hershko and Ciechanover, 1992). Modification by poly-ubiquitin often leads to protein degradation. Mono-ubiquitylation tends to regulate protein activity or acts as a signal for packaging into multivesicular endosomes (Raiborg and Stenmark, 2009; Ventii and K. D. Wilkinson, 2008). As with other post-translational protein modifications, ubiquitylation is reversible. Several classes of proteins have the ability to remove ubiquitin from specific substrates after it has been added, or to break up and recycle poly-ubiquitin chains (Hershko and Ciechanover, 1992; Ventii and K. D. Wilkinson, 2008). These enzymes are collectively called deubiquitylating enzymes, or DUBs. Deubiquitylation activity presents biochemical regulatory networks with the opportunity to temporarily modify proteins by removal of ubiquitin to recycle proteins otherwise destined for degradation, or to modulate their function. In this study, I explored the role of three putative deubiquitylating

enzymes (DUBs) in embryo polarization. These three conserved DUBs, *math-33, usp-46*, and *usp-47,* function redundantly at the genetic level with regard to their functions in polarization of *C. elegans* one-cell embryos. Based on my analysis I propose that these DUBs function during polarity establishment by contributing to positioning the centrosome, and therefore promoting cortical flow.

Methods.

Nematode Strains

Nematode strains were maintained under standard conditions (Brenner, 1974). Genetic strains used in this work are listed in Table 2.5. The *math-33(tm3561)* mutation was sequenced to find that the lesion is a frameshift that occurs at Q508 and creates a stop codon at position 510, which is within but near the end of the UCH domain.

Transgenic Strains

math-33 or *usp-47* full length cDNAs were isolated from a Gibco Proquest Library by Iproof PCR amplification (BioRad) and cloned into pIC26 (*unc-119; Ppie-1* driving a GFP-TEV-S tagged open reading frame) (Cheeseman et al., 2004). The *Ppie-1::gfp-tev-s::math-33 and Ppie-1::gfp-tev-s::usp-47* transgenic worm strains were generated by microparticle bombardment of *unc-119(ed4)* as in Praitis *et al.* 2001 (Praitis et al., 2001).

Imaging and quantification

C. elegans embryos were released from the uterus of gravid hermaphrodites with a 10 gage needle point in distilled water or in phosphate buffered saline, pH 7. Embryos were then moved onto a 2% agarose pad on a glass slide and covered with a glass cover-slip for observation.

For confocal imaging we used a Zeiss LSM 710 confocal microscope with a 63x Plan-Apochromat oil immersion lens, a stage cooled to 15°C, and Zen imaging software (Zeiss) or a Leica TCS SP2 confocal setup on a DMRE-7 microscope with a 63x oil immersion lens, and Leica imaging software. For standard wide field microscopy we used a Leica DMRA2 microscope with a 63x Leica HCX PL APO oil immersion lens, an ORCA-ER camera, and Openlab software. Live imaging experiments performed with the Leica DMRA2 were always performed in a temperature-controlled environment (16°C) to maximize the cold-sensitive phenotypes of *math-33* mutants unless otherwise noted.

Measurements from images taken by the DMRA2 were determined by measuring pixel distances using Openlab software, and calibrated to micrometers using a stage micrometer. Data from the Zeiss 710 system is calibrated, and automatically encodes distances in microns. Those distances were then measured using ImageJ. Tracing of embryo borders to measure cortical domain size was performed using Openlab. To measure domain size in this way, the circumference of the embryo cortex was traced by a "walking mouse cursor" method around the embryo. PAR-2 and PAR-3 domains were measured independently; domain boundaries were defined as the point when the cortical signal equaled the

background fluorescence. If one pole of an embryo was damaged during sample processing, that domain was not measured, but the opposite domain was measured and added to the average. Kymographs were generated with a 1 pixel-wide line through the center of the anterior-posterior axis and embryos were imaged roughly every 30 seconds.

Centrosome distance from the cortex was determined using Openlab software to measure the space between the centrosome to the closest edge of the cortex visible in the x-y plane of focus. Those centrosomes that appeared in the z plane to be very close to the bottom or the top of the embryo cortex were discarded from analysis. For Table 2.2 we measured the relative area of AB compared to the area of AB plus P1 (column 9). For this analysis we measured the circumference of blastomeres, and estimated their area from the circumference assuming a circular shaped cross-section as in (Beatty et al., 2010).

Antibody Production

GST-fusion constructs fused to a fragment of MATH-33 comprised of amino acids Q517-K706 or to USP-47 amino acids E822-M1005 in pGex-6P1 vector (GE Healthcare) were used to express protein in *E. coli* for antigen production. Proteins were eluted from glutathione-agarose beads by mixing with purified GST fusion precision 3C protease (GE Healthcare) and used to produce antibodies in guinea pigs (MATH-33), (Cocalico Biologicals) or in both guinea pigs and rabbits (USP-47), (Pocono Mountain Rabbit Farm). Affinity purification of the desired antibodies was

accomplished by adsorbing sera to antigen-agarose Hi-Trap columns (GE Healthcare), and eluting the purified antibody with 1M glycine, pH 3.

Immunohistochemistry

Embryos were processed by methanol fixation followed by incubations with primary and secondary antibodies (Guo and Kemphues, 1995). Primary antibodies used were rabbit anti-PAR-2 (Boyd et al., 1996), anti-PAR-3 mouse monoclonal (Nance et al., 2003), rabbit anti-NMY-2 (Guo & Kemphues, 1996), and mouse anti-Tubulin mouse monoclonal (a gift from Margaret Fuller) guinea pig anti-MATH-33, and rabbit anti-USP-47 (this study). Secondary antibodies used were Alexa Fluor 488 anti-rabbit, Alexa Fluor 488 anti-guinea pig (Invitrogen), FITC anti-rabbit, Cy3 anti-mouse, and Cy3 anti-guinea pig (Jackson Laboratories). Slides were mounted in Vectashield containing DAPI (4',6-diamidino-2-phenylindole). CYK-4 antibodies were a gift from Susan Mango (Jenkins, Saam, & Mango, 2006).

RNA interference (RNAi)

math-33 RNAi and *usp-47(RNAi)* was performed using the *H19N07.2* and *T05H10.1* RNAi clones from the Ahringer library (MRC Geneservice) (Kamath et al., 2003). Depletion 22 *C. elegans* DUBs was done with RNAi clones from both the Ahringer and Vidal RNAi libraries (Kamath et al., 2003; Rual et al., 2004). RNA interference of target genes was achieved by expressing dsRNA in *E. coli* followed by feeding of whole bacteria to worms (Timmons & Fire, 1998). dsRNA expression was induced with 1mM IPTG at room temperature for 3 hours, and cultures were

concentrated 10x before seeding non-nutrient agar plates. RNAi was performed at 16°C for 36-48 hours before observation and egg laying.

Phylogenetic Analysis

We identified family 25 UCH domain-containing proteins in *C. elegans* by using the ScanProsite tool from the ExPASy website of the Swiss Institute of Bioinformatics (Gattiker et al., 2002). A phylogenetic tree showing sequence relationship of UCH domains in C. elegans as well as humans and fission yeast was generated by aligning the relevant UCH domains in Megalign (Lasergene), and by plotting node distances with Phylip software (Felsenstein, 1989). We predicted closest homologs between species by using BLAST in the species of interest (NCBI) (Johnson et al., 2008). We based my assessment of USP-47's relative conservation amongst bilatera on Treefam-generated information present on the Wormbase *usp-47* gene information webpage (wormbase.org).

Results

2.1. Loss of function of *math-33* enhances weak *par* mutants

math-33 was identified in an RNA interference (RNAi)-based screen for enhancers of embryonic lethality of weak *par-1* and *par-4* mutants (D.G. Morton, personal communication) (Morton et al., 1992). *math-33* encodes a protein with a meprin and traf homology (MATH) domain, and a ubiquitin carboxy-terminal hydrolase (UCH) domain. *math-33* depletion increases the lethality of *par-1(zu310ts), par-4(it57ts),* and strongly increases lethality in three weak *par-2* mutant alleles, but not *lgl-1(tm2616)* or a partially suppressed *par-3(e2074)*

nonsense mutation (Table 2.1). I found that RNAi of *math-33* in *par-2(it5ts)* at permissive temperature resulted in polarity phenotypes at frequencies typical of strong *par-2* mutants (Table 2.2). To determine whether *math-33* has an essential role in embryonic polarity I obtained the probable null allele, *math-33(tm3561),* which truncates MATH-33 near the end of its UCH domain deleting most of the protein. I found that homozygotes for the outcrossed mutation showed maternal effect, cold-sensitive and weakly penetrant embryonic lethality, along with weakly penetrant larval lethal and sterile phenotypes (Table 2.2, Figure 2.1). I then examined embryonic polarity phenotypes in embryos in which *math-33* was mutated then depleted of *par-1* and *par-4* by RNAi. These embryos did not simply display *par-1* and *par-4* polarity phenotypes but instead resulted in synthetic phenotypes that resembled *par-2* mutants (Table 2.2). For example, whereas neither *math-33(tm3561)* nor *par-1(RNAi)* alone exhibited the characteristic *par-2* phenotype of transverse spindle orientation in P1, in combination they resulted in 71% transverse P1 spindles. Together these data indicate that *math-33* has a role in polarity.

Table 2.1.

Embryonic lethality of *par* mutants depleted of *math-33* by RNAi.

Genotype	control*(RNAi)*		*math-33 (RNAi)*	
	Embryo lethality	*n*	Embryo lethality	*n*
N2 – wild type	1%	1606	4%	1428
par-1(zu310ts)	10%	303	35%*	657
par-2(it5ts)	6%	1433	97%*	1532
par-2(it87) unc-32(e189)	8%	211	78%*	422
par-2(e2030) unc-32(e189)	0%	179	74%*	412
par-2(lw32) unc-45(e286ts)/ScI dpy-01 let-III	1%	141	1%	120
par-3(e2074); sup-7(st5)	31%	886	32%	590
par-4(it57ts)	25%	590	60%*	541
lgl-1(tm2616)	0%	301	2%	359

n= the number of embryos counted for each genotype.

* *math-33(RNAi)* lethality different from control lethality by Student's T test, $p<0.05$.

Table 2.2.

Polarity phenotypes of *math-33; par* loss-of-function embryos.

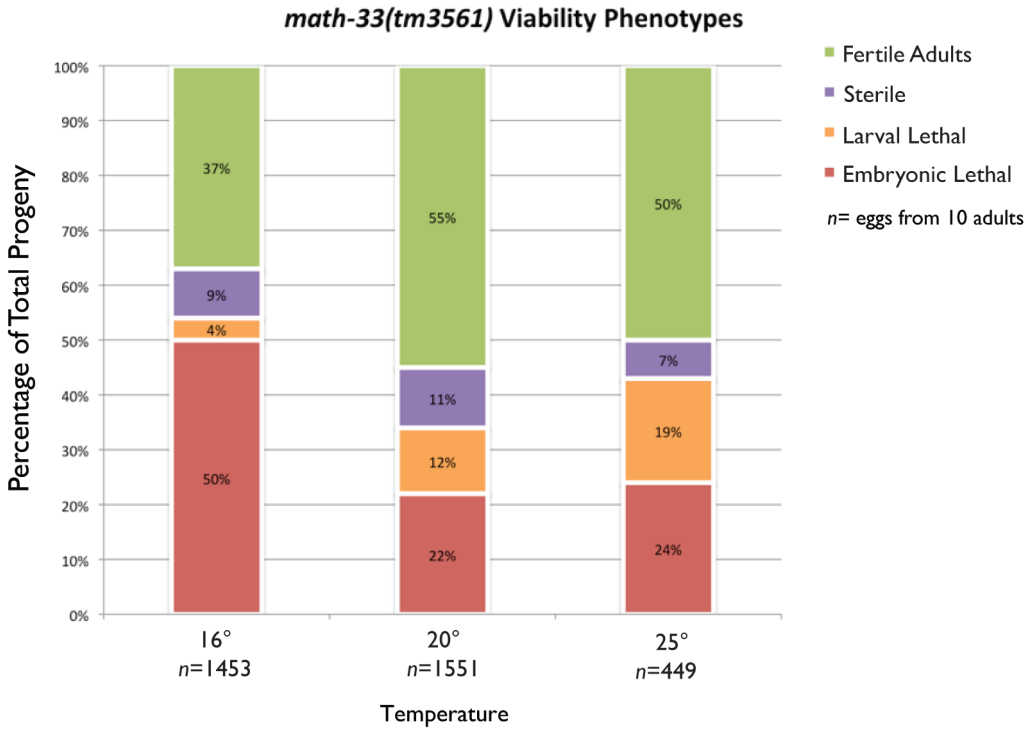
Genotype	RNAi	*n*	AB-P1 Synchrony[a]	AB-P1 Spindle Orientations				AB area[c] Total
				T-L[b]	T-T[b]	L-T[b]	L-L[b]	
N2	*control*	25	0%	100%	0%	0%	0%	57%
N2	*par-1*	14	100%	100%	0%	0%	0%	54%
N2	*par-4*	14	21%	100%	0%	0%	0%	55%
math-33(tm3561)	*control*	23	13%	100%	0%	0%	0%	57%
math-33(tm3561)	*par-1*	24	100%	29%	71%	0%	0%	53%
math-33(tm3561)	*par-4*	34	47%	88%	12%	0%	0%	53%
math-33(tm3561)	*usp-47*	12	83%	42%	50%	0%	8%	50%
par-2(it5ts)	*control*	17	12%	76%	24%	0%	0%	58%
par-2(it5ts)	*math-33*	26	96%	4%	96%	0%	0%	54%
par-3(e2074) lon-1; sup-7	*math-33*	16	25%	69%	31%	0%	0%	55%
par-3(e2074) lon-1; sup-7	*control*	17	6%	35%	29%	29%	6%	54%

[a] Proportion of embryos in which AB and P1 divided within 10 seconds of one another.

[b] Anaphase spindle orientations in two-cell embryos. T-L is the wild-type pattern with AB oriented transversely (T) and P1 oriented longitudinally (L).

[c] Average ratio of the area of an optical cross-section of the AB cell at mid-focal plane to the total embryo area in the same section (see materials and methods).

Figure 2.1.



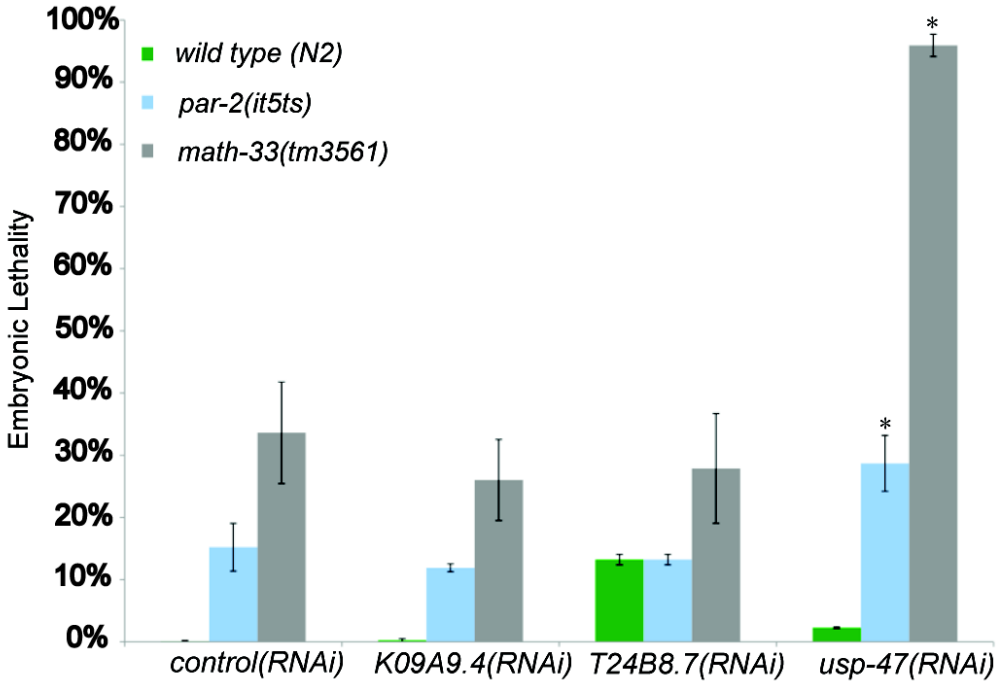
Quantification of *math-33(tm3561)* phenotypes. The proportion of *math-33(tm3561)* worms that failed to hatch (red), arrested as larvae (orange), became sterile (purple), or became fertile adults (green). The experiment was performed at 3 different temperatures on 10 whole broods each. The number of embryos obtained is given at the bottom.

2.2. The two DUBs *math-33* and *usp-47* are required for asymmetry in the one-cell embryo.

To address whether the genetic interaction between *par-2(it5ts)* and *math-33(RNAi)* is specific or whether other ubiquitin hydrolases also interact with *par-2*, I used RNAi to deplete 22 of 25 genes containing a Ubiquitin Carboxy-terminal Hydrolase (UCH) domain defining the class of DUBs to which *math-33* belongs. I found that only *math-33* RNAi depletion dramatically enhanced embryonic lethality of *par-2(it5)* at 16°. Depletion of these 22 DUBs in *math-33(tm3561)* mutants, however, revealed that depletion of *T05H10.1*, hereafter referred to as *usp-47,* caused synthetic lethality in the *math-33* mutant by increasing embryonic lethality from 35% to 95% (Figure 2.2). Furthermore, in a retest of enhancement of *par-2(it5ts)* lethality, I found that *usp-47* depletion resulted in a slight, but significant increase in lethality in *par-2(it5ts*) (15% to 28%; Figure 2.2). Two control DUBs, K09A9.4 and T24B8.7 did not significantly enhance *math-33(tm3561)* lethality. USP-47 is closely related to MATH-33 (Figure 2.3) and appears to be an out-paralog of MATH-33 that is conserved among bilatera (TreeFam), whereas MATH-33 is a more widely conserved protein found in most eukaryotes. This conservation led me to hypothesize that these two genes have overlapping functions in embryonic polarity, which I tested by examining the phenotype of *math-33(tm3561); usp-47(RNAi)* early embryos. I found that *math-33(tm3561); usp-47(RNAi)* two-cell embryos displayed variably penetrant polarity phenotypes similar to those of *par-2* mutants, including synchronous cell divisions, symmetry of cell size, and transverse spindles in both AB

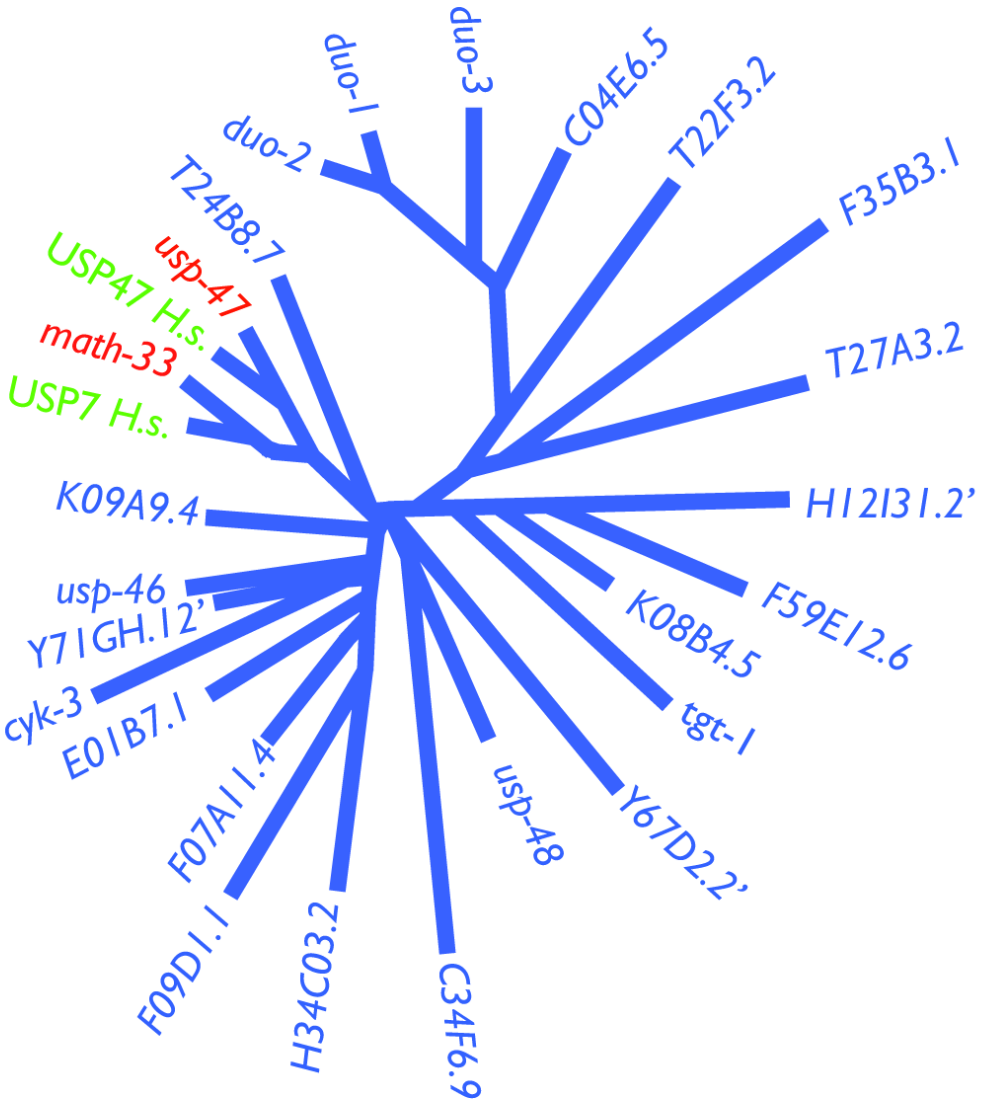
and P1, and occasional P1 cytokinesis defects (Table 2.2 and Figure 2.4). Later in my analysis, the mutant allele *math-33(ok2974)* also became available. This mutant recapitulated the *math-33(tm3561)* phenotypes by displaying a basal level of embryonic lethality, 45% that is greatly enhanced by *usp-47(RNAi)* to 93%. The rest of my analysis was performed using the *math-33(tm3561)* allele.

Figure 2.2.



usp-47(RNAi)* increases embryonic lethality of *par-2(it5ts)* and *math-33(tm3561). Bars indicate the % embryonic lethality. A minimum of *n= 300* embryos was scored for each genotype in two trials. Error bars indicate standard error of the mean, and asterisks indicate significance relative to controls in a Student's T-test at $p < 0.01$. K09A9.4 and T24B8.7 served as negative control DUBs that we found do not enhance *math-33(tm3561)* embryonic lethality.

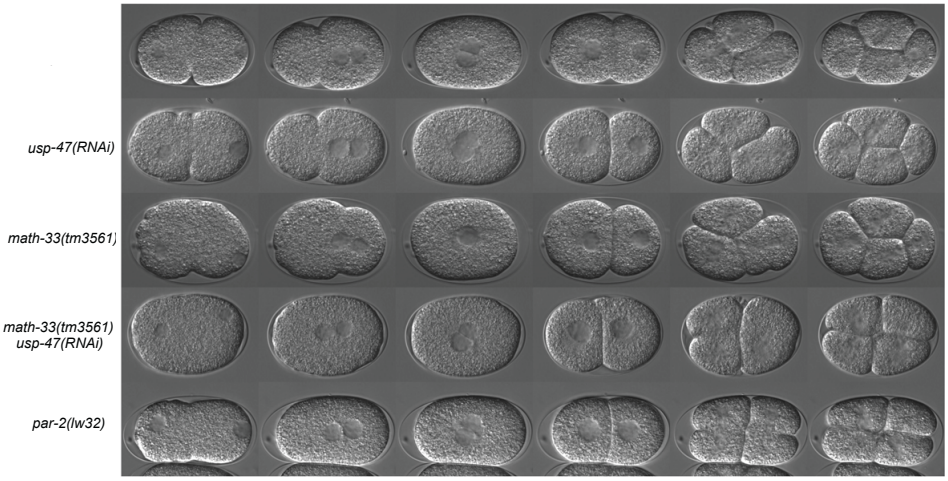
Figure 2.3.



Phylogenetic tree comparing *C. elegans* UCH domains. Un-rooted phylogenetic tree of 25 UCH domains in *C. elegans* and 2 human homologs of *math-33* and *usp-47*. I did not perform RNAi on the three genes indicated by '. Human homologs USP7

and USP47 are included to highlight that there is evolutionary conservation of the DUBs between species.

Figure 2.4.

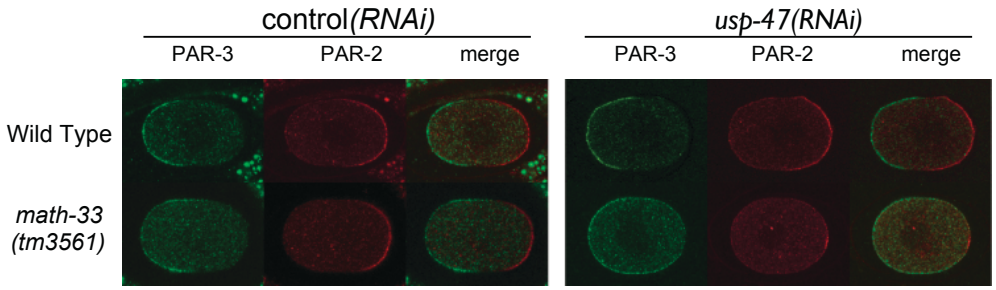


DIC time-lapse of one-cell to four-cell embryos of the indicated genotypes. Embryos are oriented with the anterior at the left in this and subsequent figures. The spindle orientation of P1 as it divides is usually longitudinal to the embryo axis, but is transverse in polarity defective mutants such as *par-2(lw32).* These embryos were observed at room temperature.

I hypothesized that the simultaneous loss of MATH-33 and USP-47 would affect the distribution of PAR-2 and PAR-3 at the cell cortex. One-cell embryos immunostained for PAR-2 and PAR-3 and scored between onset of pronuclear migration and centration indicate that *math-33(tm3561); usp-47(RNAi)* embryos exhibit a variable decrease in the size of the posterior cortical domain, and a reciprocal increase in the size of the anterior cortical domain (Figure 2.5, 2.6). This result illustrates that the loss of the two DUBs causes defects in PAR protein distributions at the one-cell stage. To determine the basis for the smaller posterior domain marked by PAR-2, I examined *math-33(tm3561); par-2::gfp; usp-47(RNAi)* embryos by time-lapse video microscopy, and observed two phenotypic classes (Figure 2.7). In class I, PAR-2::GFP was recruited weakly to the cortex in a domain whose size was comparable to wild type and was maintained through the first cleavage. These embryos went on to divide normally. In class II, PAR-2::GFP was initially recruited to a smaller domain, which failed to expand and did not persist through cell division. These class II embryos exhibited polarity defects at first and second cleavages (Figure 2.7). To determine whether there was a reciprocal effect on the anterior PAR domain, I attempted to construct a *math-33(tm3561)* strain expressing PAR-6::mCherry. Unfortunately, perhaps due to the over-expression of PAR-6, I was unable to maintain *math-33(tm3561); par-6::mCherry* worms in stock. However, *lgl-1::GFP; par-6::mCherry; math-33(tm3561)* worms are relatively healthy, perhaps because LGL-1::GFP expression counteracts the PAR-6::mCherry. Depletion of USP-47 in this strain resulted in a clearing of PAR-6::mCherry during establishment that was less robust than in wild type (Figure 2.8). The result that

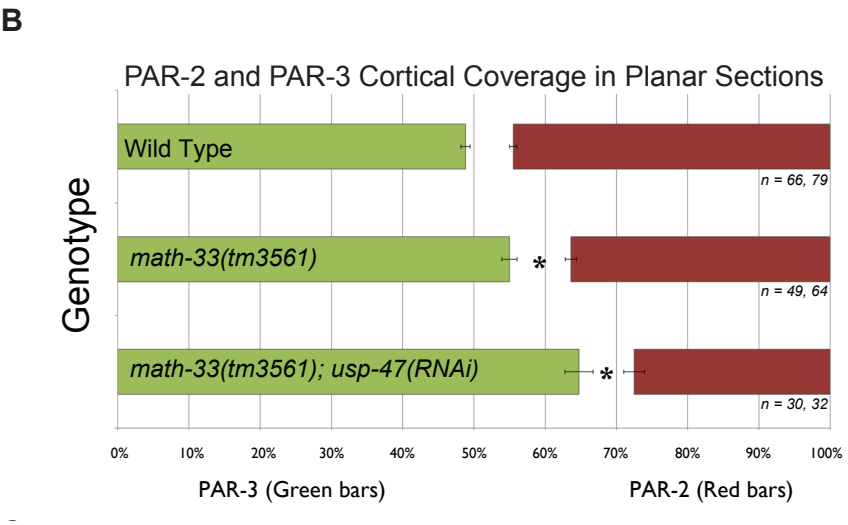
both PAR-6 clearing and PAR-2 localization in the posterior are impaired suggests that the establishment of a posterior domain is compromised in the absence of MATH-33 and USP-47.

Figure 2.5.



PAR-3 occupies a larger portion of the cortex in *math-33(tm3561); usp-47(RNAi)* embryos. PAR-3 (green), PAR-2 (red) showing examples of PAR-2 and PAR-3 distribution in controls and an embryo showing a *math-33(tm3561); usp-47(RNAi)* embryo in which PAR-3 occupies a large region of the cortex.

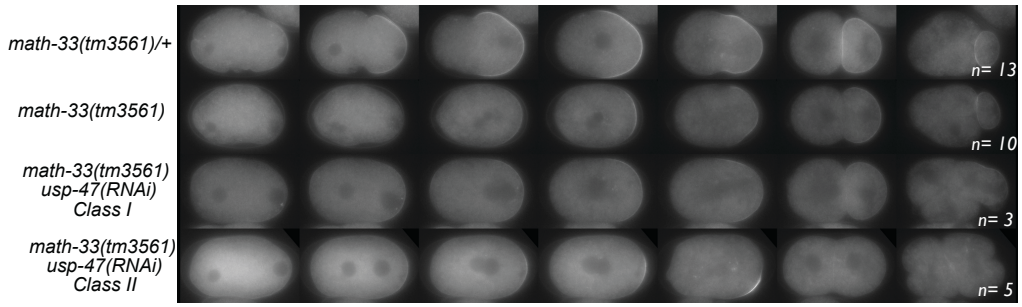
Figure 2.6.



Averages of PAR-3 and PAR-2 domain sizes in immunostained embryos.

Domain size as a percentage of embryo length. *n* indicates the number of anterior and posterior domains examined respectively.

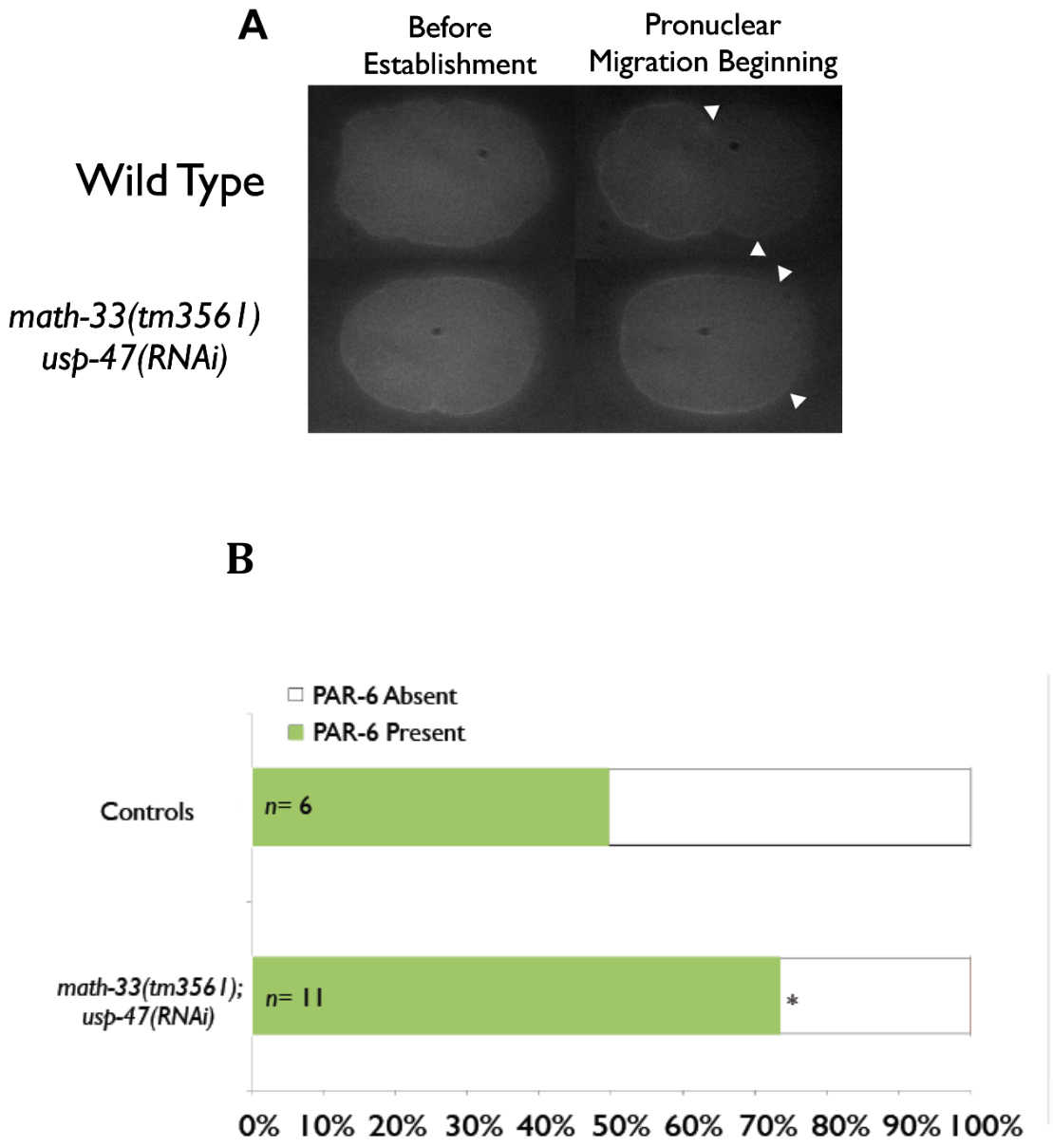
Figure 2.7.



Time-lapse images displaying the dynamic localization of PAR-2::GFP.

Compared to controls *math-33(tm3561); usp-47(RNAi)* embryos weakly recruit PAR-2 and could be grouped into two classes: those that maintain a PAR-2 domain throughout the cell cycle (class I), and those in which the weak PAR-2 domain is not maintained (class II). Representative embryos are shown.

Figure 2.8.



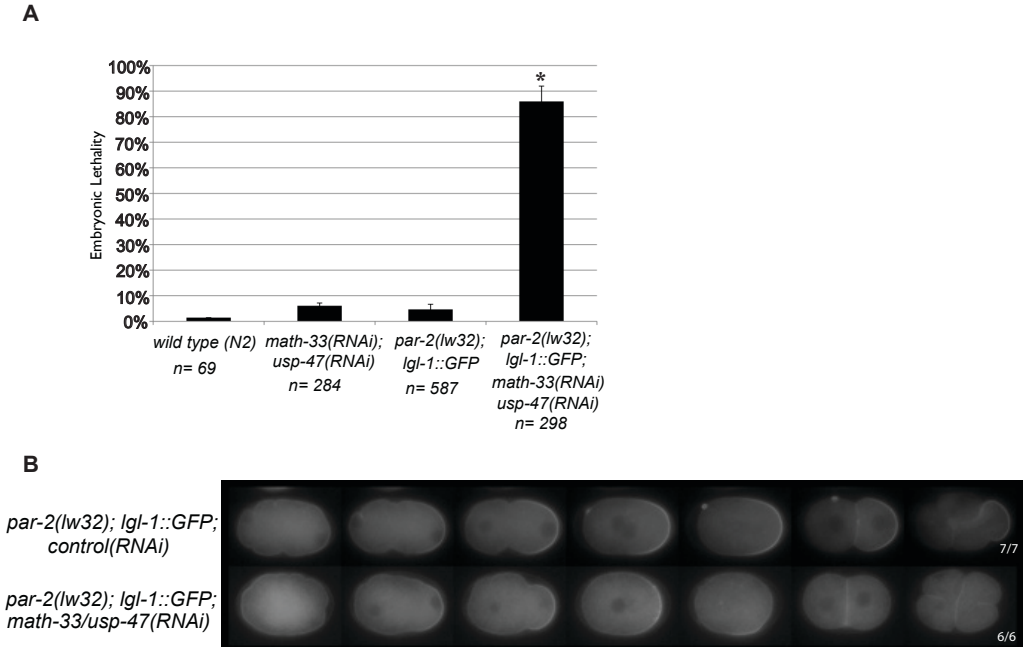
PAR-6 clearing from the posterior is reduced in *math-33(tm3561); usp-47(RNAi).* (A) PAR-6::mCherry localization prior to establishment and after establishment. White arrowheads indicate the extent to which PAR-6 is absent from the posterior cortex. (B) Measurement of the maximum PAR-6::mCherry clearing

from the posterior as a proportion of the total cortex. PAR-6::mCherry cleared less in *math-33(tm3561); usp-47(RNAi)* embryos $p < 0.05$.

2.3. *math-33* and *usp-47* control polarity independently of PAR-2.

The sequence similarities between MATH-33 and USP-47 (Figure 2.3) and their shared role in polarity raise the possibility that these two proteins act to deubiquitylate common substrates. PAR-2 is a putative E3 ubiquitin ligase that may self-regulate through auto-ubiquitylation (Y. Hao et al., 2006). Since *math-33(tm3561); usp-47(RNAi)* embryos resemble *par-2* mutants, I hypothesized that ubiquitylated-PAR-2 could serve as a regulatory target of the DUBs. To test this, I attempted to examine PAR-2 protein levels in embryo protein extracts, but were not able to obtain interpretable results. Instead, I tested genetically to determine whether the DUBs were required in a genetic background that was null for PAR-2, but in which embryos develop normally through over-expression of *LGL-1::GFP* (Beatty et al., 2010). I depleted *math-33* and *usp-47* simultaneously in *par-2(lw32); lgl-1::GFP* and found that depletion of the DUBs caused 86% embryonic lethality (Figure 2.9A). Embryos displayed defects in the ability of LGL-1::GFP to remain stable at the posterior cortex, and had transverse mitotic spindles in 6/6 P1 blastomeres (Figure 2.9B). I interpret these results to mean that the two DUBs do not act exclusively through PAR-2 to control polarity.

Figure 2.9.

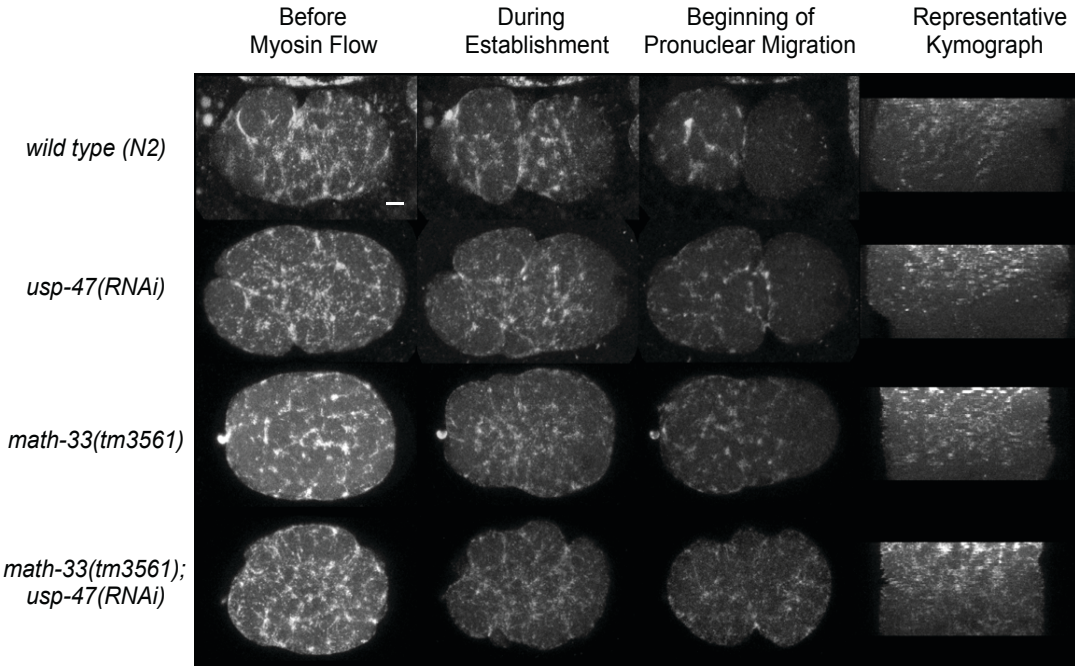


PAR-2 function is not required to mediate loss of polarity in *math-33(RNAi) usp-47(RNAi).* (A) Embryonic lethality of the indicated genotypes. (B) LGL::GFP time-lapse of individual embryos. The localization of LGL::GFP was monitored over time. In the absence of DUBs, the LGL::GFP domain size is not maintained similar to previous results such as with PAR-2::GFP in Figure 2.7, and transverse spindles occur in P1.

2.4. MATH-33 and USP-47 are required for cortical flows in the one-cell embryo.

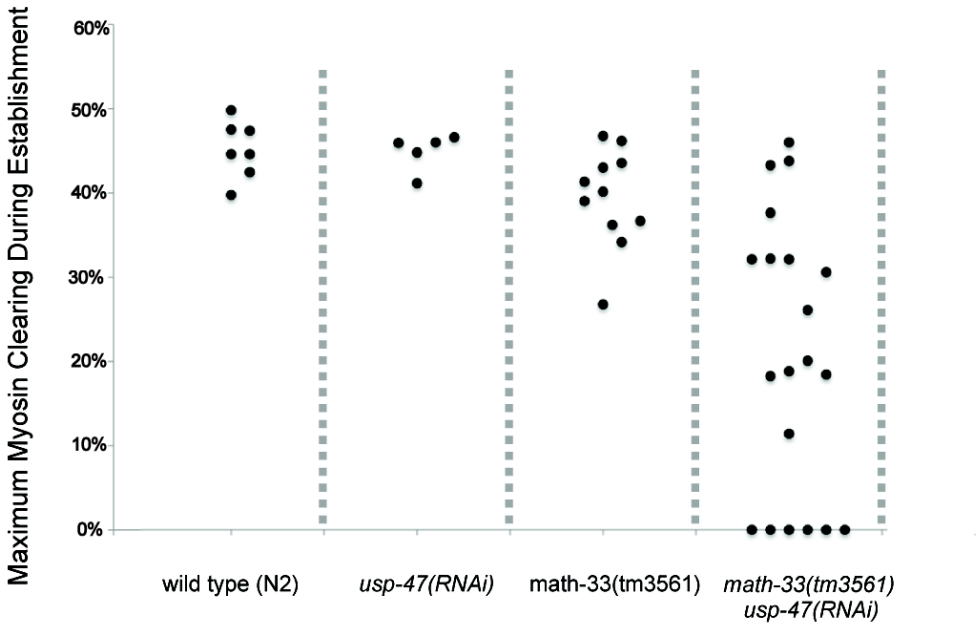
Careful examination of *math-33(tm3561); usp-47(RNAi)* embryos revealed that in addition to *par-2*-like phenotypes, cortical contractility is decreased, embryos frequently lack pseudocleavage (Figure 2.4), exhibit low penetrance cytokinesis defects (fewer than 2 in 10 embryos in most experiments), and have occasional polar body extrusion defects (not quantified). The lack of pseudocleavage and cytokinesis in this subset of embryos led me to hypothesize that absence of the DUBs may lead to defects in actomyosin function, which could lead to the failure to establish a posterior domain. To test this hypothesis, I observed cortical NMY-2 (type II non-muscle myosin heavy chain) in 20 *math-33(tm3561); nmy-2::gfp; usp-47(RNAi)* embryos by confocal live imaging (Figure 2.10) and found that contractile myosin foci are present, but that the extent of myosin flow from the posterior to the anterior was variably defective. Embryos showed a range in the extent of myosin clearing, with 4/20 embryos showing normal clearing, 6/20 embryos with no clearing and 10/20 embryos with intermediate levels of clearing (Figure 2.11). I confirmed this result by observing endogenous NMY-2 by immunostaining. Three out of seven *math-33(tm3561); usp-47(RNAi)* embryos at post-pronuclear decondensation showed no evident clearing of myosin foci compared to none of six controls (Figure 2.12). Thus actomyosin-dependent cortical flow is defective in *math-33(tm3561); usp-47(RNAi)* embryos.

Figure 2.10.



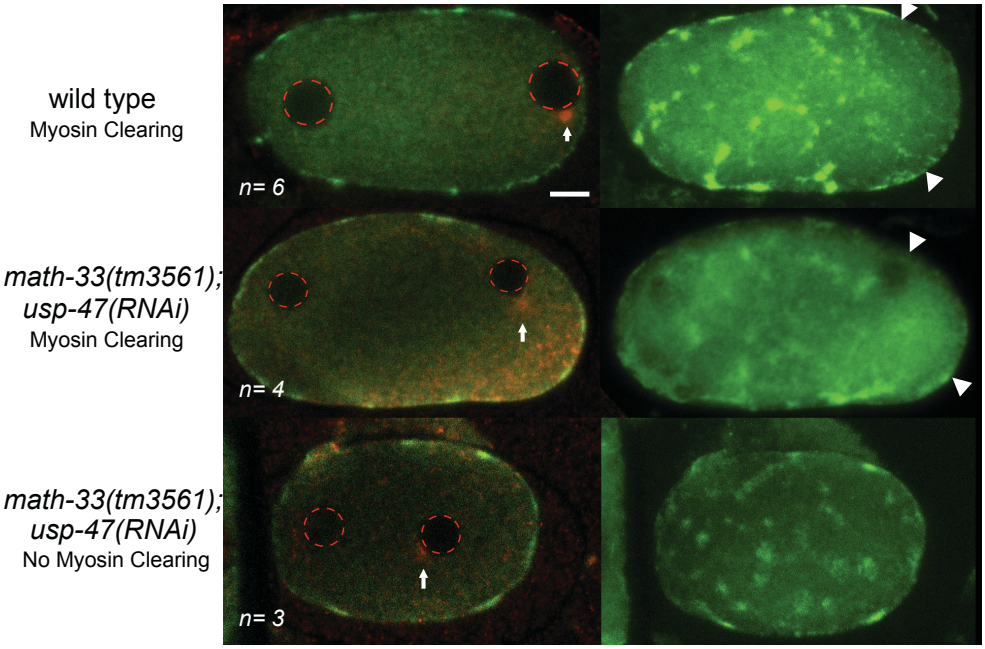
The posterior to anterior myosin flow is defective in math-33(tm3561); usp-47(RNAi). Time-lapse confocal images of embryos expressing NMY-2::GFP. The maximum projections of sections through the cortex of the embryo show the localization of NMY-2::GFP foci at three stages. Kymographs are shown to illustrate the pattern of myosin clearing and flow over time. About halfway through the kymographs (from top to bottom), large myosin foci transitioned into small myosin puncta.

Figure 2.11.



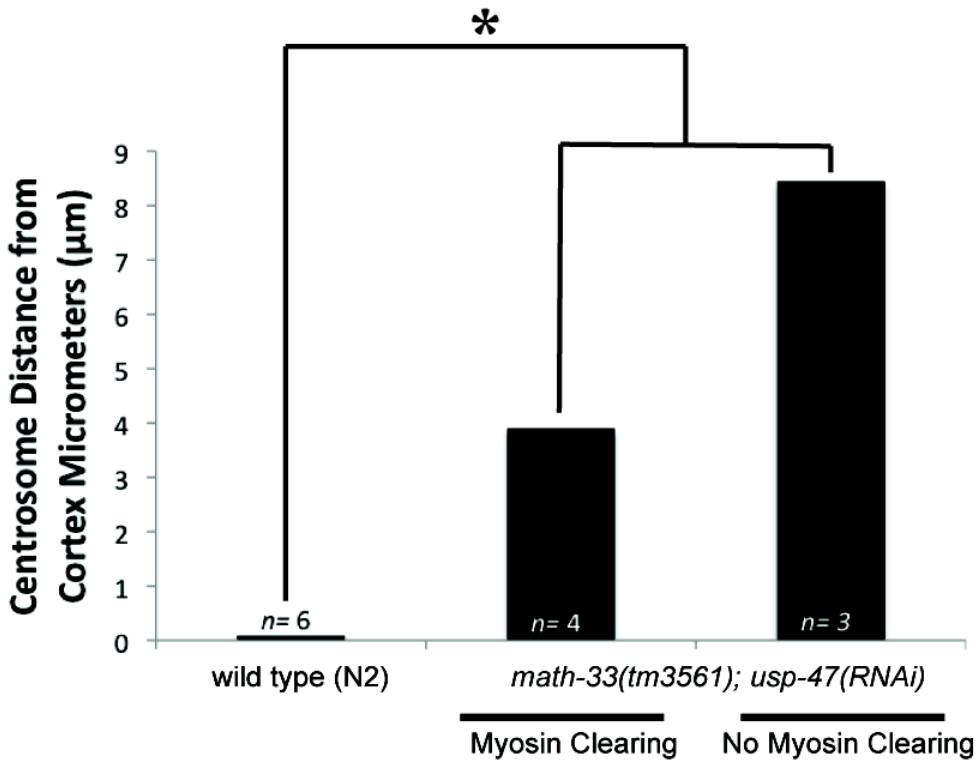
NMY-2 maximum clearing from the posterior. Myosin move from the posterior to the anterior creating a clearing, and the maximum extent of this clearing was measured as a proportion of the entire embryo length. Each dot represents an embryo of the indicated genotype.

Figure 2.12.



Confocal stacks of embryos immunostained for NMY-2 (green) and tubulin (red). Arrows indicate the location of the centrosome, red dotted circles mark the position of pronuclei, and the extent of myosin clearing is indicated by arrowheads.

Figure 2.13.



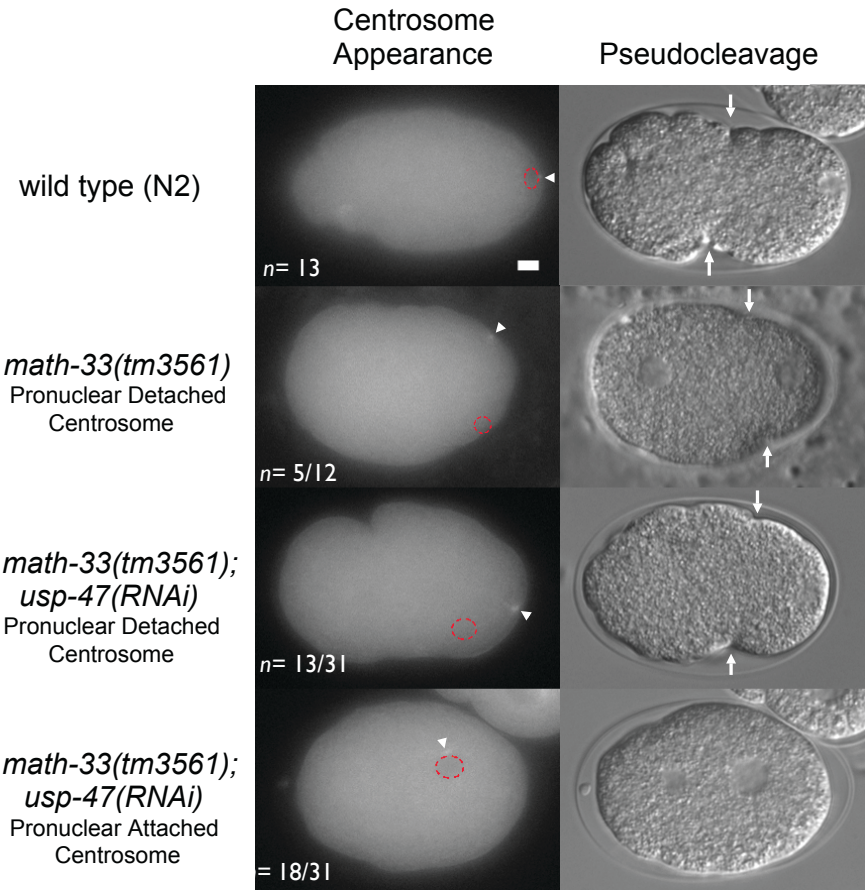
Centrosome position in fixed embryos. For embryos examined in Figure 2.12, the average centrosome distance is significantly different than N2 controls $p < 0.01$.

2.5. MATH-33 and USP-47 promote attachment of the centrosome to the cortex and to the male pronucleus.

Because cortical flow is dependent upon a signal from the centrosome (Munro et al., 2004), I examined the position of the centrosome in fixed embryos immunostained with NMY-2 (Figure 2.12). In this small data set I noted that the centrosome appeared to be significantly more distant from the cortex in *math-33(tm3561); usp-47(RNAi)* embryos (Figure 2.13) and that this correlated with the extent of myosin clearing. However, because fixed embryos do not provide sufficient temporal resolution, I examined centrosome behavior in live embryos using tubulin::GFP to follow centrosomes. In control embryos the centrosome is closely apposed to the cortex and the pronucleus when the tubulin::GFP signal is first detectable (Figure 2.14, 2.15, 2.23). In *math-33(tm3561); tub-2::gfp; usp-47(RNAi)* embryos the position of the centrosome with respect to both the pronucleus and the cortex was variably abnormal. I observed two phenotypic classes with respect to pronuclear attachment. In some embryos, at first detection, the centrosome was attached to the male pronucleus (*n*= 18/31 from multiple experiments, Figure 2.14, 2.15, 2.23). In the other embryos the centrosome was detached from the pronucleus (*n*= 13/31 from multiple experiments, Figure 2.14, 2.15, 2.23). The centrosome was also detached from the pronucleus in 5/14 *math-33(tm3561); tub-2::gfp* embryos. There is no statistical difference in the number of detached centrosomes between *math-33(tm3561)* and *math-33(tm3561); usp-47(RNAi).* However, I did observe significant differences in the centrosome's position relative to the cortex when first detectable (Figure 2.15). I also noted that when the centrosome was attached to the male pronucleus, it was more likely to be far from the cortex (Figure 2.15). In all 13

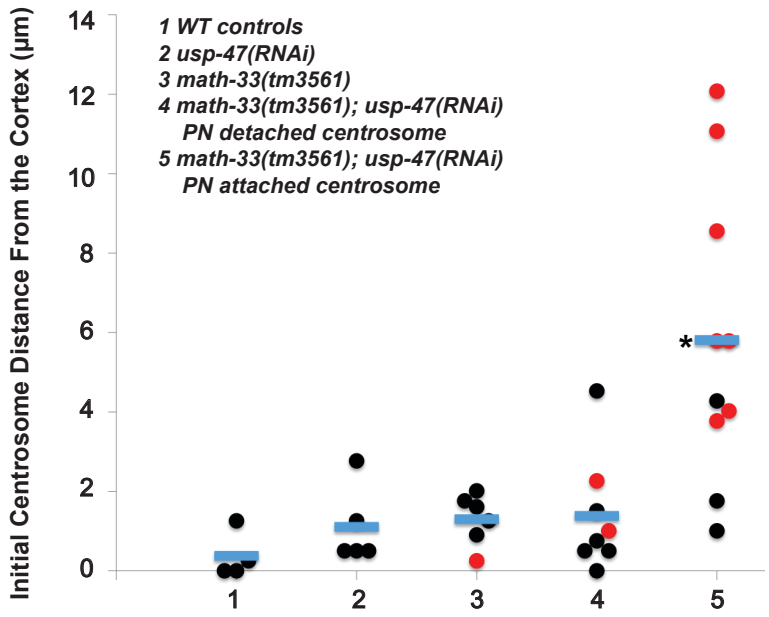
cases in which centrosomes were initially detached from the male pronucleus, the centrosome and pronucleus migrated toward one another and re-associated. To determine whether the initial distance of the centrosome from the cortex correlated with polarity defects in this assay, I examined pseudocleavage (PC), an indirect indicator of myosin flow and contractility, in these same embryos. In 17/31 embryos in which pseudocleavage was detectable, the distance of the centrosome from the cortex was on average 2.1 μm, whereas in 14/31 embryos lacking detectable pseudocleavage, the average distance was significantly larger, 7.0 μm (Figure 2.18, embryos lacking PC are red dots in 2.15 and 2.23). I found this live imaging data to be consistent with immunostaining experiments (Figure 2.12, 2.13). Because of this correlation I hypothesized that centrosome mis-localization may be responsible for the weak myosin flows.

Figure 2.14.



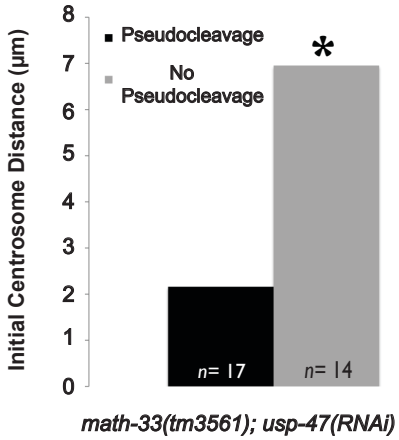
The DUBs regulate the position of the centrosome in early one-cell embryos. Micrographs showing tubulin::GFP in embryos at the time the centrosome was first detected. Centrosomes are marked with a white arrowhead and the position of the paternal pronucleus is indicated by red dotted circles. The DIC micrograph column shows representative embryos of the same genotypes at the time of pseudocleavage (an indirect indicator of the extent of myosin clearing). The white arrows indicate the location of pseudocleavage furrows.

Figure 2.15.



The centrosome is more distant from the cortex in embryos lacking the DUBs. The distance in micrometers of the centrosome from the embryo cortex at the time of earliest detection for the indicated genotypes. Each data point represents a single embryo. Black dots are embryos that had normal or weak pseudocleavage, red dots are embryos that displayed no pseudocleavage. Blue bars mark the mean distance $p < 0.01$ for column 1 vs. 5.

Figure 2.16



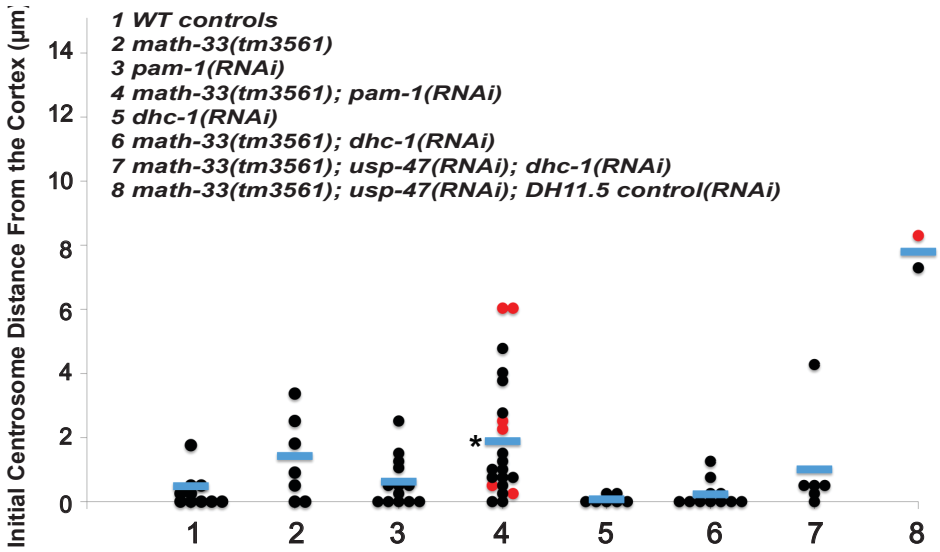
Pseudocleavage defects increase as centrosome distance increases. Embryos were pooled from five RNAi experiment replicates. Differences were statistically significant according to a T test; $p < 0.01$.

To test this hypothesis I used *dhc-1(RNAi)* to deplete dynein, which causes centrosomes to strongly localize at the cortex (Fortin et al., 2010), and scored polarity phenotypes and centrosome behavior. I reasoned that if the defect in cortical association was causing the polarity defect, then forcing a tight association of the centrosome would suppress the polarity defects. Depletion of *dhc-1* in both *math-33(tm3561)* and math-33(tm3561); *usp-47(RNAi)* resulted in a closer initial association of the centrosome to the cortex (Figure 2.17). I also found that 10/10 *math-33(tm3561); usp-47(RNAi); dhc-1(RNAi)* embryos displayed robust pseudocleavage, whereas 4/7 control *math-33(tm3561); usp-47(RNAi*) *DH11.5(RNAi)* had no or weak pseudocleavage (Figure 2.18). Thus, influencing the centrosome position to bring it closer to the cortex suppresses *math-33; usp-47(RNAi)* polarity phenotypes, consistent with the hypothesis that the primary effect of the DUBs on polarity is through centrosome positioning.

The gene *pam-1* is known to affect centrosome dynamics in the early embryo (Lyczak et al., 2006; Fortin et al., 2010) in a way that is similar, but not identical to, the loss of the DUBs. Loss of *pam-1* does not appear to affect the initial proximity of the centrosome to the cortex, but rather results in premature departure of the centrosome from the cortex (Lyczak et al., 2006). To test for possible genetic interaction between *pam-1* and *math-33*, I examined centrosome behavior and pseudocleavage in *math-33(tm3561); pam-1(RNAi)* embryos expressing tubulin::GFP. I found that *pam-1(RNAi)* enhanced centrosome defects in *math-33* in a way similar to *usp-47(RNAi)*. Relative to *math-33(tm3561)* or *pam-1(RNAi)* alone, I noted an increase in the fraction of centrosomes that were located further from the

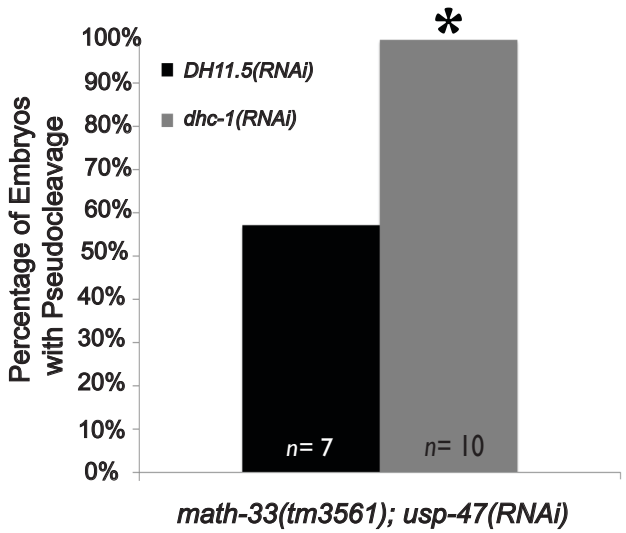
cortex (Figure 2.17), an increase in the frequency of centrosomes detached from the nucleus, as well as more frequent pseudocleavage defects (red dots in Figure 2.17).

Figure 2.17.



Depleting dynein or PAM-1 influences the centrosome position in *math-33* embryos. The distance of the centrosome from the cortex measured in the indicated genetic backgrounds. $p < 0.01$ for column 4 compared to WT distances from multiple experiments, but $p = 0.156$ for column 4 compared *to math-33(tm3561)* in multiple experiments.

Figure 2.18

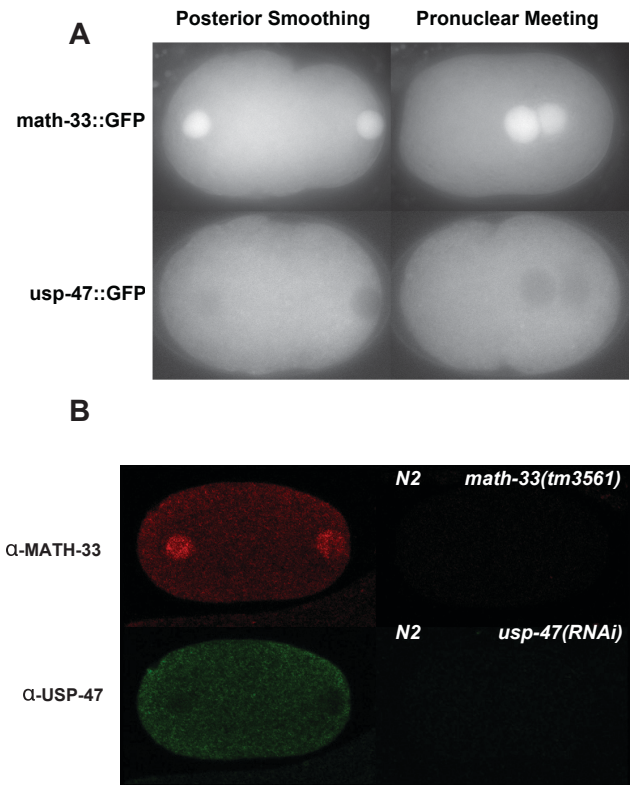


Depletion of dynein suppresses centrosome localization defects. A single experiment to examine pseudocleavage defects caused by DUBs. Pseudocleavage defects are completely suppressed by *dhc-1(RNAi). p* < 0.05.

2.6. MATH-33 and USP-47 are expressed in early embryos and the germline.

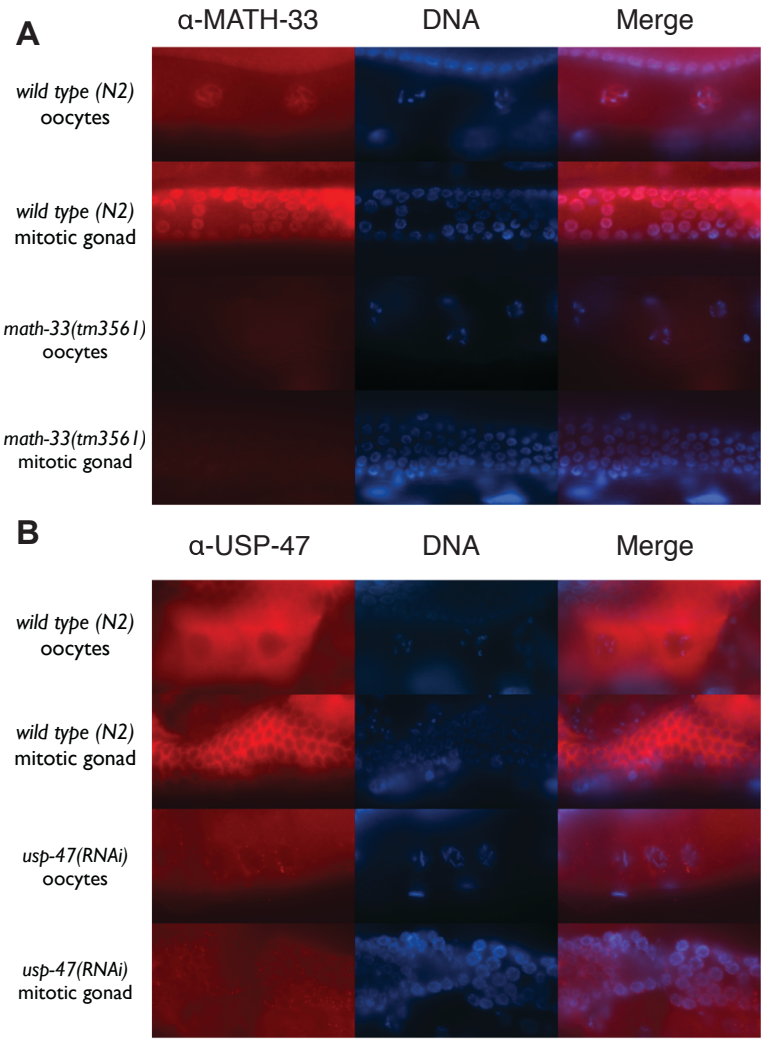
In an effort to gain insight into possible targets of MATH-33 and USP-47, I examined expression patterns and localization of MATH-33 and USP-47 in embryos and adult worms. I generated anti-MATH-33 and anti-USP-47 antibodies and *pie-1* promoter driven MATH-33::GFP and USP-47::GFP transgenic worm strains. The antibodies and GFP fusions gave consistent results in early embryos: MATH-33 is present at high levels in both cytoplasm and nucleus, whereas USP-47 is only detected at high levels in the cytoplasm (Figure 2.19). Furthermore, antibody staining shows that MATH-33 is present in most or all cells in the worm (not shown) and is enriched in the germline (Figure 2.20A), whereas USP-47 is present primarily in the germline (Figure 2.20B). MATH-33 foci that do not overlap with condensed chromatin can also be seen in oocyte nuclei (Figure 2.21A). Enrichment of MATH-33 and USP-47 in the germline led me to hypothesize that germline or early embryo is the major site of action of the protein. MATH-33::GFP protein is expressed by the *pie-1* promoter so that MATH-33::GFP is maternally packaged into embryos (Mello et al., 1992). Expression of *Ppie-1::math-33::GFP* in the germline of *math-33(tm3561)* mutants was able to suppress lethality and sterility phenotypes (Figure 2.1), indicating that the maternal contribution of MATH-33 to embryos is sufficient to compensate for most essential MATH-33 functions. If MATH-33 and USP-47 act redundantly, these localization data suggest that the cytoplasm is the primary compartment of the early embryo in which these two proteins are required for their polarity functions.

Figure 2.19.



MATH-33 and USP-47 are not enriched at cortex or centrosome. (A) Representative embryos showing the distribution of GFP::MATH-33 and GFP::USP-47 in time-lapse. Both proteins are present in the cytoplasm, but MATH-33 is enriched in nuclei. (B) Co-immunostaining of endogenous MATH-33(red) in the cytoplasm and the nucleus; USP-47(green) primarily in the cytoplasm. Negative controls lacking either MATH-33 or USP-47 have reduced levels of staining indicating the antibodies are specific.

Figure 2.20.



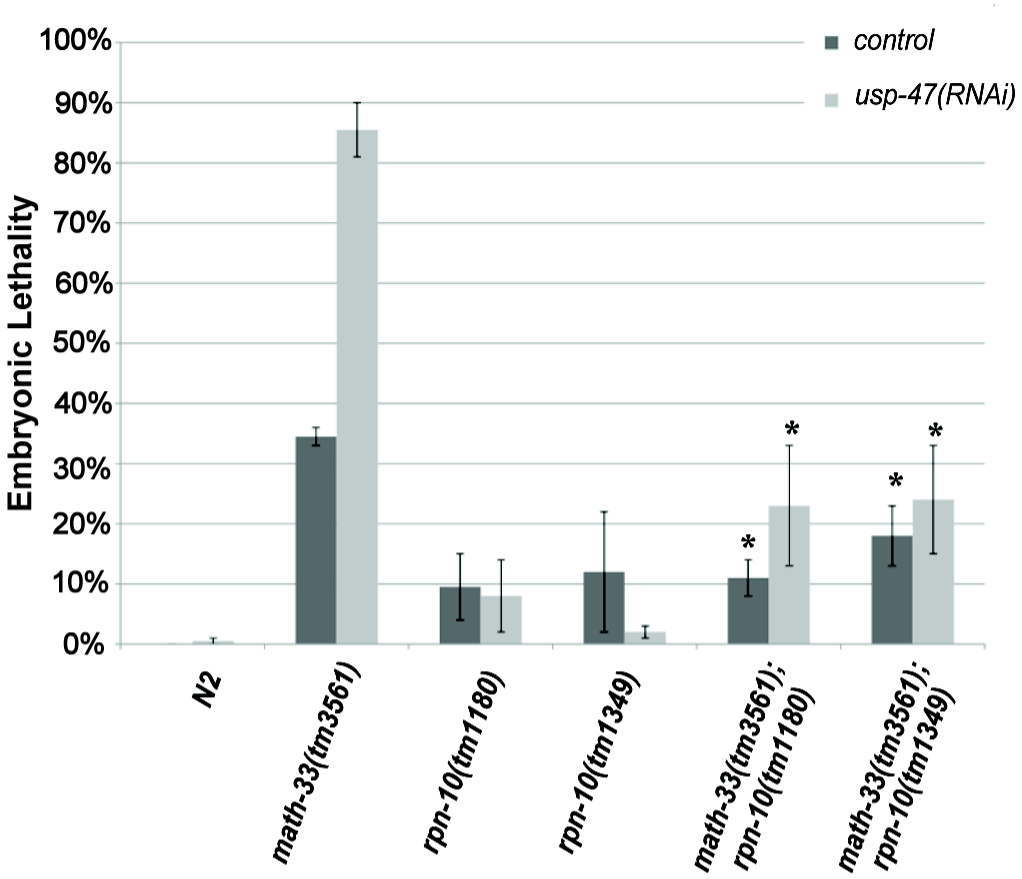
MATH-33 and USP-47 are present in the germline. Micrographs showing oocytes and a mitotic portion of the gonad for each indicated genotype. (A) Worms immunostained for MATH-33 (red), DAPI (blue), and an overlay (red & blue). (B) Worms immunostained for USP-47 (red), DAPI (blue), and an overlay.

2.7. MATH-33 and USP-47 Antagonize Protein Degradation.

The presence of a UCH domain in MATH-33 and USP-47 suggest that these proteins act as deubiquitylation enzymes. Removal of ubiquitin could have different consequences: a) prevention of degradation, b) modulation of protein activity c) effects on endocytosis or recycling of membrane proteins (Ventii and K. D. Wilkinson, 2008; Raiborg and Stenmark, 2009). I tested whether the major action of MATH-33 and USP-47 was to antagonize poly-ubiquitylation by asking whether compromising proteasome activity could suppress the lethality and polarity defects of *math-33(tm3561); usp-47(RNAi)* worms. I compromised protein degradation by crossing *rpn-10(tm1349)* or *rpn-10(tm1180)* into *math-33(tm3561)* worms. RPN-10, like its baker's yeast homolog, is considered to be a non-essential ubiquitin recognition protein that acts to bring substrates to the proteasome to increase the rate at which the substrates are degraded (Labbé et al., 2006). *rpn-10* mutation has been shown to affect sex determination (Shimada et al., 2006) and to suppress phenotypes of *par-2(it5ts*) (Labbé et al., 2006). My results show that *rpn-10* mutations strongly suppress *math-33(tm3561); usp-47(RNAi)* embryonic lethality and polarity phenotypes (Figure 2.22, 2.23). Because compromising the proteasome could affect polarity in a number of ways, I asked whether *rpn-10* mutations also suppressed the centrosome positioning defects in *math-33; usp-47(RNAi)* embryos. I found that centrosomes in *rpn-10(tm1349); math-33(tm3561); usp-47(RNAi)* embryos were indeed always found very close to the cortex, similar to wild type

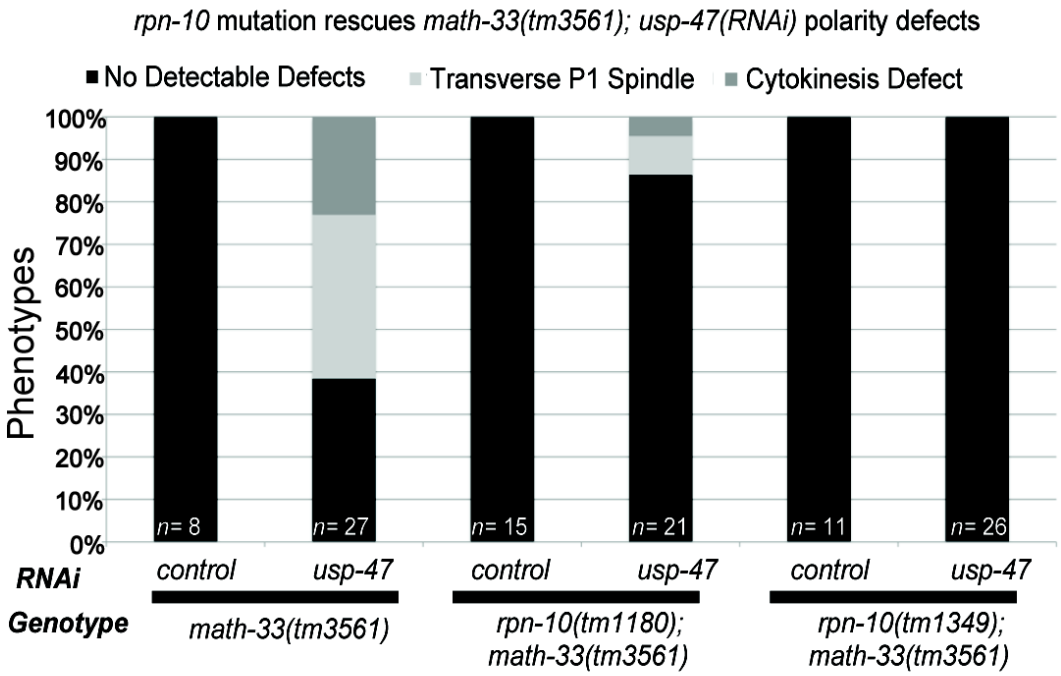
(Figure 2.23). Therefore, I conclude that in their role in early embryonic polarity, the DUBs regulate protein turnover.

Figure 2.21.



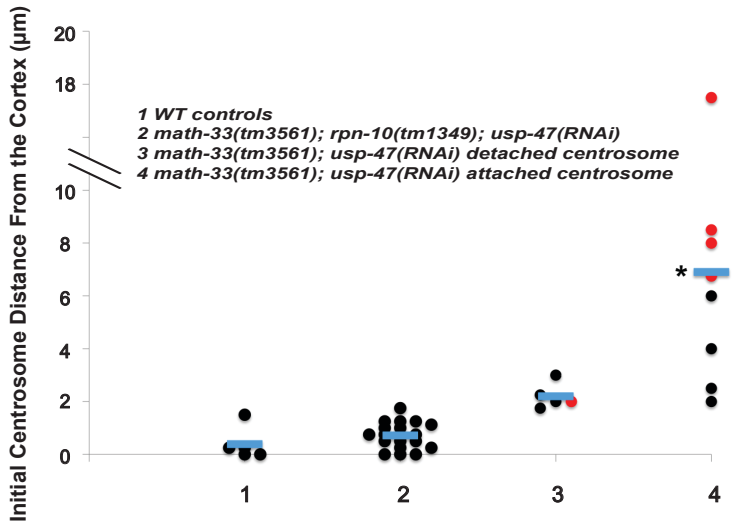
Mutation of rpn-10 suppresses embryonic lethality in math-33(tm3561); usp-47(RNAi) embryos. The embryonic lethality of *math-33(tm3561); usp-47(RNAi)* is reduced by either of two *rpn-10* mutations. s.e.m is indicated by the error bars $n >$ 350 embryos for N2 controls and $n > 600$ in the other cases.

Figure 2.22.



Mutation of *rpn-10* suppresses transverse P1 spindle defects in *math-33(tm3561); usp-47(RNAi)* embryos. A single experiment examining the penetrance of transverse P1 spindles and cytokinesis defects in P0. Two phenotypes were measured, Transverse P1 spindles and cytokinesis defects in P1. The frequency of these defects for each genotype is indicated. The two *rpn-10* alleles did not behave identically in the experiment, which may be because *rpn-10(tm1349)* is a larger deletion in the *rpn-10* gene.

Figure 2.23.



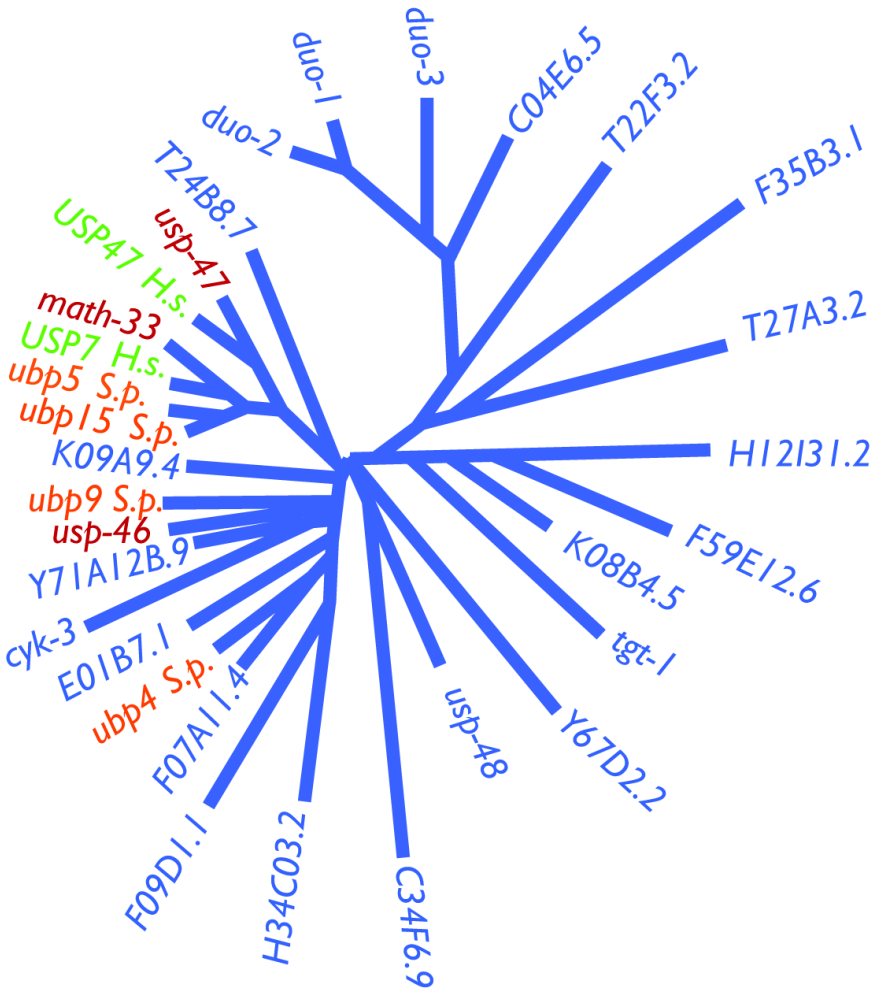
Mutation of *rpn-10* suppresses centrosome localization defects in *math-33(tm3561); usp-47(RNAi)* embryos. Distance in micrometers of the centrosome from the embryo cortex when it is first detectable. *rpn-10* mutation is able to suppress all pseudocleavage defects, and the localization of the centrosome relative to *math-33(tm3561); usp-47(RNAi)* controls. None of the 18 centrosomes observed in column 2 were detached from the paternal pronucleus indicating that the detachment phenotype was also completely suppressed. Column 4 was significantly different $p < 0.01$ compared to column 1 or 2.

2.8. USP-46 also functions redundantly with MATH-33 and USP-47.

A recent paper from Kouranti *et al.,* identified a novel and redundant role for a group of five DUBs, including homologs of *math-33* and *usp-47*, in polarity of *Schizosaccharomyces pombe* (Kouranti et al., 2010). Their results suggested to me that the role of this class of deubiquitylases in polarity could be evolutionarily conserved. The yeast proteins Ubp15p and Ubp5p are homologs of MATH-33 and USP-47 (Figure 2.24). Because of the link between the yeast DUBs and polarity, I hypothesized that homologs of (*ubp4, ubp9*), the two other *S. pombe* UCH-containing DUBs that affect polarity in *S. pombe*, might also function in polarity in *C. elegans*. *C. elegans usp-46* and *E01B7.1* are homologs of the yeast proteins *ubp9* and *ubp4*, respectively (Table 2.3). RNAi depletion of neither gene increases embryo lethality in wild type or in *math-33(tm3561)* (data not shown). However, a deletion mutant allele *usp-46(ok2232)* in combination with simultaneous *math-33(RNAi)* and *usp-47(RNAi)* resulted in 75% embryonic lethality (*n= 1330*), compared to 30% lethality (*n= 1408) in math-33(RNAi); usp-47(RNAi)* in wild type. Of 20 *usp-46(ok2232)* embryos in which *math-33* and *usp-47* were simultaneously depleted, 11 displayed transverse P1 spindle orientations (Figure 2.25) compared to 1/30 *math-33(RNAi); usp-47(RNAi)* in wild type. However, *math-33(tm3561); usp-46(ok2232)* double mutants are largely sterile, producing only a few oocytes and no fertilized eggs, raising the possibility that the two genes act redundantly in gametogenesis. After mating to wild-type males, the *math-33(tm3561); usp-46(ok2232)* mutants can produce a few fertilized embryos which fail to hatch. Five of the six one-cell

embryos I were able to obtain in this way displayed transverse P1 spindle orientations at the two-cell stage (Figure 2.25) indicating defects in polarity. More recently a new deletion mutation, *usp-47(tm4950),* became available; I found that double mutants of *usp-47(tm4950); usp-46(ok2232)* are unhealthy and display mild embryonic lethality and sterility. At 16°, these double mutants have no evident polarity defects, but at 25° some embryos display transverse spindles in P1 (4/18, Table 2.4), while the single mutants do not. *usp-46; usp-47* mutant embryos depleted for *math-33* display a high penetrance of transverse spindle orientations (11/15; Table 2.4), and embryos appear to be similar in most respects to *math-33(tm3561); usp-47(RNAi)* embryos (see Figure 2.4). Taken together, the increasing penetrance of polarity phenotypes as a function of loss of activity of the three deubiquitylases (Table 2.4) suggests that the three enzymes function redundantly in early polarity, but that they vary in their contributions to the polarization process.

Figure 2.24.



Phylogenetic *math-33, usp-46 and usp-47* UCH domains relative to *S. pombe* polarity DUBs. An un-rooted phylogenetic tree of UCH domains aligned by amino acids. *usp-47C.e.* and *math-33C.e.* are a short distance on the tree from homologs *USP47H.s., USP7H.s., ubp5S.p.,* and *ubp15S.p.* indicating that their UCH domains share more homology than other *C.e.* UCH domains.

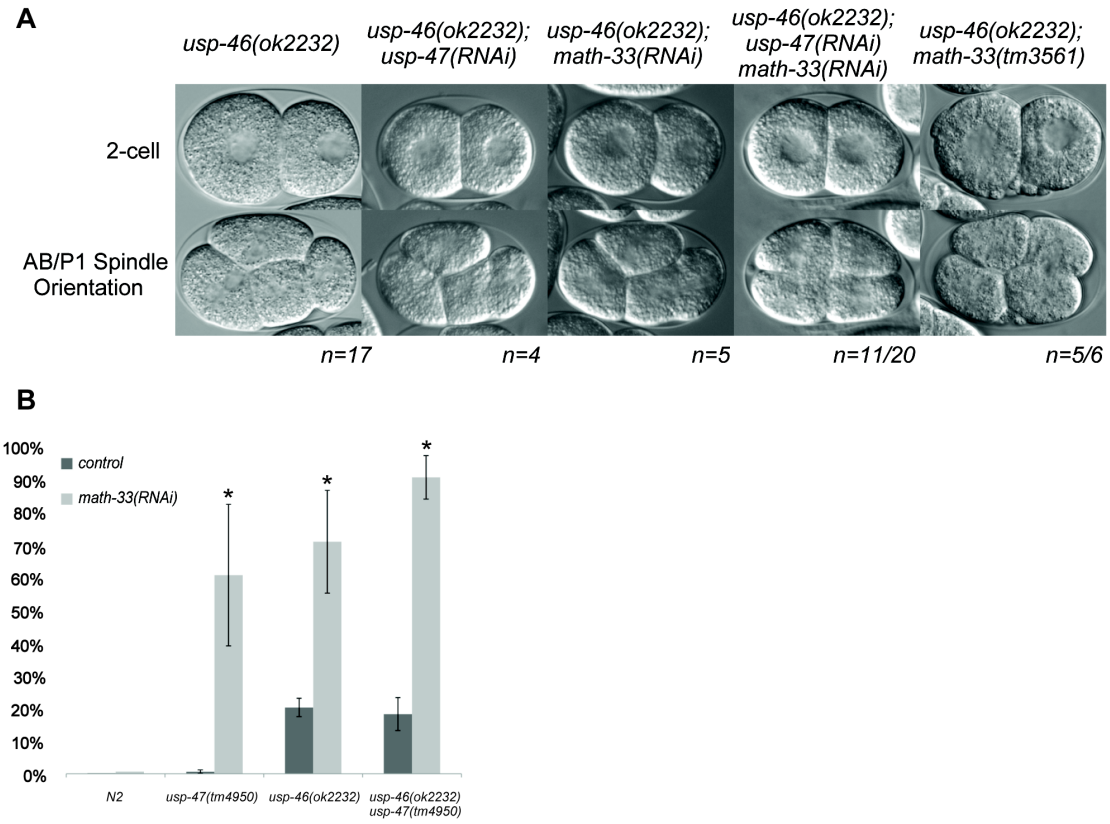
Table 2.3.

Homologous DUBs in several species.

Schizosaccharomyces pombe	*Caenorhabditis elegans*	*Drosophila melanogaster*	*Homo sapiens*
ubp4	*E01B7.1*	*CG14619*	*USP8*
ubp5	*math-33*	*USP7*	*USP7*
ubp9	*usp-46*	*CG7023*	*USP12*
ubp15	*math-33*	*USP7*	*USP7*
sst2	*F37A4.5*	*CG2224*	*STAMBP*
ubp5	***usp-47***	*USP-64E*	*USP47*

The closest homologs of the five *S. pombe* DUBs involved in asymmetry of endocytosis. Sequences of DUBs from *S. pombe* were used as a basis to find the next closest homologs in *C. elegans, Homo sapiens,* or *Drosophila melanogaster.* The BLAST seed sequence for each row is indicated in bold.

Figure 2.25.



***usp-46* loss affects embryonic lethalty and P1 spindle orientation.** (A) Two-cell embryos at interphase (top) and P1 mitosis (bottom) to illustrate unequal vs. equal first divisions and spindle orientations. Maternal genotypes are indicated. (B) Embryonic lethality measured after depleting *math-33* in mutants for *usp-46* and *usp-47.*

Table 2.4.

Effect of RNAi/mutant combinations of *math-33, usp-46,* and *usp-47* on P1 spindle orientation.

Temperature	Genotype	T-T	*n*
16°	*N2*	0%	25
16°	*usp-46(ok2232)*	0%	25
16°	*usp-47(tm4950)*	0%	13
25°	*usp-46(ok2232)*	0%	9
25°	*usp-47(tm4950)*	0%	9
25°	*math-33(tm3561)*	0%	23
16°	*usp-46(ok2232); usp-47(tm4950)*	0%	23
16°	*N2; usp-47(RNAi) math-33(RNAi)*	3%	30
25°	*usp-46(ok2232); usp-47(tm4950)*	22%	18
16°	*math-33(tm3561); usp-47(RNAi)*	64%	28
16°	*usp-46(ok2232); usp-47(RNAi) math-33(RNAi)*	65%	20
16°	*usp-46(ok2232); usp-47(tm4950); math-33(RNAi)*	73%	15
16°	*usp-46(tm3561); math-33(tm3561)*	83%	6

T-T indicates that both AB and P1 cells of two-cell stage embryos divided transversely.

Discussion.

Ubiquitin regulation appears to have an important but not well-understood role in *C. elegans* embryonic polarization. One of the earliest discoveries was that PAR-2 has homology to RING domain E3 ubiquitin ligases, suggesting that ubiquitin ligase activity may be important for excluding anterior PARs from the posterior (Levitan et al., 1994; Boyd et al., 1996). Hao and colleagues showed that the PAR-2 RING domain is required for robust transgene rescue of embryos lacking endogenous PAR-2, indicating that it is likely to be an active ubiquitin ligase *in vivo*, although this activity is not absolutely essential for function (Hao et al., 2006). Biochemical targets of PAR-2, however, are unknown. Other results that relate ubiquitin-based regulation to polarization include the finding that PAR-6 levels are affected by activity of the ubiquitin ligase CUL-2 and its adapter protein FEM-3 (Pacquelet et al., 2008), and that mutations of *C. elegans* homologues of the BRAT family of ubiquitin ligases have been shown to be able to suppress weak *par-2* phenotypes (Hyenne et al., 2008). Weak impairment of the proteasome through mutation of *rpn-10* has also been shown to suppress *par-2* phenotypes (Labbé et al., 2006), and depletion of the proteasome regulatory subunit, *rpn-2* results in abnormal spindle orientation in AB at the two-cell stage (Davy et al., 2001; Nocker et al., 1996).

I report here additional evidence for an important role of ubiquitylation in embryonic polarity. I show that a group of three putative deubiquitylating enzymes, MATH-33, USP-46 and USP-47, contribute to polarity establishment in *C. elegans,*

likely in a redundant fashion. Analysis of *math-33(tm3561); usp-47(RNAi)* embryos revealed that when the products of these two genes are missing or reduced, PAR protein domains are abnormally sized, cortical actomyosin flow is weak or fails, and centrosomes are variably positioned with respect to the cell cortex. Because distance of centrosomes from the cortex correlates with the severity of the polarity phenotypes, and because blocking movement of the centrosome away from the cortex by depleting dynein heavy chain restores polarity establishment in *math-33(tm3561); usp-47(RNAi),* I propose that the primary role of the DUBs in polarity is to promote the association of the centrosome and the cortex.

Prior to polarization in the wild-type *C. elegans* embryo, the centrosome at first detection can be observed within a 0-9 *u*m range (5 *u*m average) from the cortex (Cowan and Hyman, 2004; Bienkowska and Cowan, 2012). It then moves to within 0-4 *u*m at the time of polarity initiation (Cowan and Hyman, 2004; Bienkowska and Cowan, 2012). It has been observed that asymmetry can still weakly occur even when the centrosome remains distant from the cortex but when the centrosome is closer to the cortex this process is much more efficient (Bienkowska & Cowan, 2012). In embryos lacking functional MATH-33 and USP-47 the position of the centrosome when first detected is often distant from the cortex and these unassociated centrosomes remain so. Therefore, I suggest that forces that maintain close association of the centrosome and cortex are weakened or absent and as a result, polarity establishment as assessed by myosin flow is either weak or non-existent. However, I also see that centrosomes are often detached from the paternal pronucleus, indicating that the pronuclear-centrosomal interaction is

defective. Centrosome-nuclear attachment is in many cases unnecessary for the proper localization of centrosomes in cells (Reinsch and Gönczy, 1998), and indeed I see that detached centrosomes can associate closely with the cortex. Therefore it is likely that the DUBs affect proteins required for the centrosome to interact with the cortex and pronucleus. Indeed, it appears that weakened associations create a competition between cortex and nucleus for binding to the centrosome – centrosomes that are detached from the nucleus are more likely to be closely apposed to the cortex and centrosomes that are attached to the paternal pronucleus are more likely to be more distant from the cortex. The detachment of the centrosome from the pronucleus must occur between the time that the embryo is fertilized and when tubulin::GFP first allows the centrosome to become visible. Furthermore, all detached centrosomes are capable of re-attaching to the paternal pronucleus in a manner similar to maternal pronuclear capture by the growing astral microtubules. This suggests that a microtubule-mediated tracking mechanism (Reinsch and Gönczy, 1998) in which pronuclei migrate towards the centrosome on astral microtubules functions in a relatively normal way. Because of this, I speculate that the early centrosomal attachment to the pronucleus uses a different mechanism than the microtubule-based mechanism that promotes attachment during prophase.

The establishment phase of polarity is certainly affected by the loss of MATH-33 and USP-47. However I also observed distinct defects in polarity maintenance such as loss of PAR-2::GFP and LGL::GFP domains at the posterior cortex after prophase. The loss of maintenance is probably caused by re-entry of the anterior PARs into the posterior domain and could occur for two reasons. One is that *math*-

33(tm3561); usp-47(RNAi) causes the initial clearing of myosin to be impaired, and thus the initial size of the posterior domain to be smaller. Afterwards, maintenance could fail due to the inability of posterior PARs to exclude anterior PARs. A second possibility is that the DUBs actively participate in maintaining the posterior domain. Therefore it is possible that the DUBs have important roles during both the establishment and maintenance phases, although most of the evidence I present argues for a stronger role in establishment.

The DUBs appear to act by protecting proteins from degradation rather than by modifying protein activities or localizations. Evidence for this is that *rpn-10* mutations are effective suppressors of *math-33(tm3561); usp-47(RNAi)*. RPN-10 likely acts as it does in other organisms to recognize ubiquitylated substrates at the proteasome, and therefore mutating *rpn-10* may compromise efficacy of protein degradation at the proteasome (Finley et al., 1996; Davy et al., 2001). *rpn-10* mutation corrects the centrosome localization at the cortex as well as to the pronuclear envelope. In contrast, depletion of dynein heavy chain suppresses the defect in centrosome-cortex association, and polarity defects, but does not suppress pronuclear envelope detachment, indicating that phenotypic suppression by depleting dynein bypasses the normal role of the DUBs. Thus, I propose that cortical association and pronuclear attachment are regulated by maintaining an appropriate level of one or more key centrosomal proteins. Impaired function of the centrosome is the simplest explanation for the phenotypes observed when the DUBs are absent. However, several other possibilities exist. For example, regulatory proteins that in turn regulate the function of the centrosome could be targets of the DUBs.

Other proteins are also known to be required for proper interaction of the centrosome with the cortex in one-cell *C. elegans* embryos: dynein components and regulators, such as dynein heavy chain, mentioned above (Cockell et al., 2004) and a puromycin sensitive aminopeptidase (PAM-1) (Fortin et al., 2010; Lyczak et al., 2006). However, reduction or loss of function of neither the dynein group nor PAM-1 precisely mimics the phenotypes I describe here. In embryos severely depleted of dynein heavy chain, 15% of centrosomes can become detached from the pronucleus, and in most embryos the centrosomes become tightly and persistently associated with the cortex (Fortin et al., 2010). Thus a key function of dynein is to positively promote dissociation of the centrosome from the cortex. In *pam-1* mutant embryos the centrosome-pronuclear complex is correctly positioned in the posterior at the cortex, but the complex spends less time at the posterior, leaving early, leading to polarity establishment defects (Fortin et al., 2010; Lyczak et al., 2006). Because both the centrosome association and pronuclear attachment are mildly enhanced in *math-33(tm3561);pam-1(RNAi)* embryos it seems likely that the two proteins affect both processes; my experiments, however, do not allow me to distinguish whether the DUBs and PAM-1 act through a common pathway or affect centrosome dynamics through two separate pathways. A proposed function of PAM-1 is that it may remove N terminal peptides from proteins to allow them to be ubiquitylated (Lyczak et al., 2006). If this is the case, it is not clear how the DUBs would work with PAM-1 in a common pathway.

Comparison of my results with results from studies of homologous proteins in the fission yeast *Schizosaccharomyces pombe* raises the possibility that these

DUBs have a conserved role in eukaryotic cell polarity. A screen in *S. pombe* of DUB function showed that *ubp4, ubp5, ubp9, ubp15,* and *sst2,* although non-essential individually, act redundantly to affect asymmetric endocytosis (Kouranti et al., 2010). Because this group contained two proteins homologous to MATH-33 and USP-47, Ubp5p and Ubp15p, I speculated that if the functions were evolutionarily conserved, homologs of the other members of the group might have redundant roles in *C. elegans*, and led me to the discovery of a role for USP-46, the homolog of UBP9p, in *C. elegans* embryos. In *S. pombe,* microtubules are required to control proper polar growth (Siegrist and Doe, 2007; Martin, 2009), so there may be an underlying common mechanism that involves the centrosome. Alternatively, since Kouranti *et al.* (Kouranti et al., 2010) noted strong phenotypes related to endocytosis, it may be possible that membrane proteins in *C. elegans* that contribute to cytoskeletal regulation are regulated by the DUBs.

The DUBs I examined appear to have both overlapping and distinct functions that may extend to diverse biological processes. *math-33(tm3561)* mutants display mild polarity phenotypes, whereas *usp-46(ok2232)* and *usp-47(RNAi)* do not. This indicates *math-33* individually plays a more crucial role in regulating *C. elegans* polarity than the other two DUBs. It is unlikely, however, that *math-33's* role is limited to early embryo polarity establishment. After nine outcrosses, *math-33(tm3561)* mutants remain pleiotropic, displaying embryonic lethality, larval lethality, and sterility. Furthermore, *math-33* has been reported to interact genetically with *vab-10, ksr-1,* and *skn-1* in *C. elegans* (Kahn, Rea, Moyle, Kell, & Johnson, 2008; Rocheleau et al., 2008; Zahreddine, Zhang, Diogon, Nagamatsu, &

Labouesse, 2010) indicating that it is likely to act in several biological pathways that may not relate directly to polarity. MATH-33 homologs in other organisms provide little insight on an exact mechanism of action in polarity, but suggest that the homologous DUBs have diverse functions. In *Saccharomyces cerevisiae,* the *math-33* homolog *UBP15* causes mislocalization of the cell membrane protein Gap1 to cytoplasmic membranes, and results in lower permease activity (Costanzo et al., 2010). In mammals, the *math-33* homolog *USP7/HAUSP* has been studied extensively for its ability to bind and deubiquitylate *p53* and the ubiquitin ligase *MDM2,* and it has also been implicated as a negative regulator of PTEN localization to the nucleus (Li et al., 2004; Sheng et al., 2006; Song et al., 2008). USP7 was also found to co-immunoprecipitate with the PAR-1 homolog MARK4 (Brajenovic, Joberty, Küster, Bouwmeester, & Drewes, 2004) but does not appear to be able to deubiquitylate it (Al-Hakim et al., 2008). USP7 is also considered to be a therapeutic target for cancer therapy due to its broad role in genomic stability (Colland et al., 2009). In contrast, there is less known about the roles of *usp-46* and *usp-47* in other organisms. In my study I found that the individual loss of either *usp-47* or *usp-46* have no discernible phenotypes in early *C. elegans* embryos. *usp-47* has not previously been studied, but *usp-46* has been shown to have deubiquitylation activity, and to regulate the levels of GLR-1 abundance in a fashion that suggests USP-46 deubiquitylation of GLR-1 on endosomes prevents degradation in the multivesicular body/lysosome pathway (Kowalski, Dahlberg, & Juo, 2011). It is possible then that *usp-46* and *usp-47* may have biological roles that are redundant with other DUBs, and as a result have fewer obvious phenotypes than *math-33*.

In summary, I have identified three deubiquitylase genes, *math-33*, *usp-47 and usp-46* that are required for proper polarity establishment in *C. elegans*. The enzymes appear to act by stabilizing proteins that promote the interaction of the centrosome with the cell cortex. As a result, this thesis provides evidence that a new and unique set of genes relating to ubiquitin-regulation have a specific and unknown targets that are necessary for development. In part, I argue that the target pathway of the DUBs may not just be important for *C. elegans* but also eukaryotes. A key to determining the truth of such an argument will be decided by determining the targets of these deubiquitylases with respect to their role in early embryonic polarity. I have cursorily explored this in Appendix B. It is possible that future research in cell biology, and *C. elegans* will connect the mechanism of the DUBs to the nature of polarity establishment.

Table 2.5.

Strains used in this study.

N2	wild type
AZ235	*unc-119(ed3) III; ruls48[pAZ147 Ppie-1::tbb-2::gfp, unc-119(+)]* (Praitis et al., 2001)
FX04954	*usp-47(tm4954) II*
FX2616	*lgl-1(tm2616) X* (Beatty et al., 2010)
JJ1473	*unc-119(ed3) III; zuls45[nmy-2::nmy-2::gfp+unc-119(+)] V*. (Nance et al., 2003)
KK83	*par-2(e2030) unc-32(e189) III* (Kemphues et al., 1988)
KK196	*par-3(e2074)III; sup-7(st5) X* (Kemphues et al., 1988)
KK300	*par-4(it57ts) V* (Morton et al., 1992)
KK574	*par-2(it87) unc-32(e189) III* (Morton et al., 1992)
KK747	*par-2(lw32) unc-45(e286ts)/ sC1 III* (Levitan et al., 1994)
KK810	*par-1(zu310ts) V*
KK879	*itIs153 [Ppie-1::par-2::gfp] II; par-2(lw32) unc-45(e286ts) III*
KK1033	*par-2(it5ts) III* – outcrossed to N2 one time from KK418 (Kemphues et al., 1988)
KK1042	*itIs272[Ppar-6::par-6::mCherry, unc-119(+); unc-119(ed4) III* (K. Basch, H. Kim, K. Kemphues unpublished)
KK1056	*math-33(tm3561) V* – outcrossed to N2 nine times from RB2194
KK1064	*itIs288[Ppie-1::gfp-tev-s::math-33 +unc-119(+)]; unc-119(ed4) III*
KK1069	*math-33(tm3561) V / nT1[qIs51] myo-2::gfp pes-10::gfp F22B7.9::gfp (IV:V)*
KK1070	*itIs153[Ppie-1::par-2::gfp] II; math-33(tm3561) V / nT1[qIs51] myo-2::gfp pes-10::gfp F22B7.9::gfp (IV:V)*
KK1091	*unc-119(ed4) III; math-33(tm3561)V; itIs288[Ppie-1::gfp::math-33, unc-119(+)]*
KK1094	*unc-119(ed3) III; math-33(tm3561)V; ruls48[pAZ147 Ppie-1::tbb-2::gfp, unc-119(+)]*
KK1098	*math-33(tm3561) zuls45[Pnmy-2::nmy-2::GFP, unc-119(+)] V*
KK1119	*par-2(lw32) unc-45(e286ts) III; itIs256[Plgl-1::lgl-1::gfp, unc-119(+)] V* (Beatty et al., 2010)
KK1135	*math-33(tm3561) V; itIs256[Plgl-1::lgl-1::gfp, unc-119(+)]; itIs272[Ppar-6::par-6::mCherry, unc-119(+)]*
KK1136	*itIs290[Ppie-1::gfp-tev-s::usp-47 +unc-119(+)]; unc-119(ed4) III*
KK1138	*rpn-10(tm1180) I; math-33(tm3561) V*
KK1139	*rpn-10(tm1349) I; math-33(tm3561) V*
KK1141	*unc-119(ed3) III; math-33(tm3561) V; ruls48[pAZ147P pie-1::tbb-2::gfp, unc-119(+)]*
KK1174	*rpn-10(tm1349) I; math-33(tm3561)V; ruls48[pAZ147 Ppie-1::tbb-2::gfp, unc-119(+)]*
KK1180	*usp-46(ok2232) III* – outcrossed to N2 three times

RB2194 *math-33(ok2974) V*

UM25 *rpn-10(tm1349) I* (Labbé et al., 2006)

UM26 *rpn-10(tm1180) I* (Labbé et al., 2006)

Appendix

Appendix A. The localization and composition of fibrous body membranous organelles is affected in *math-33(tm3561); usp-47(RNAi).*

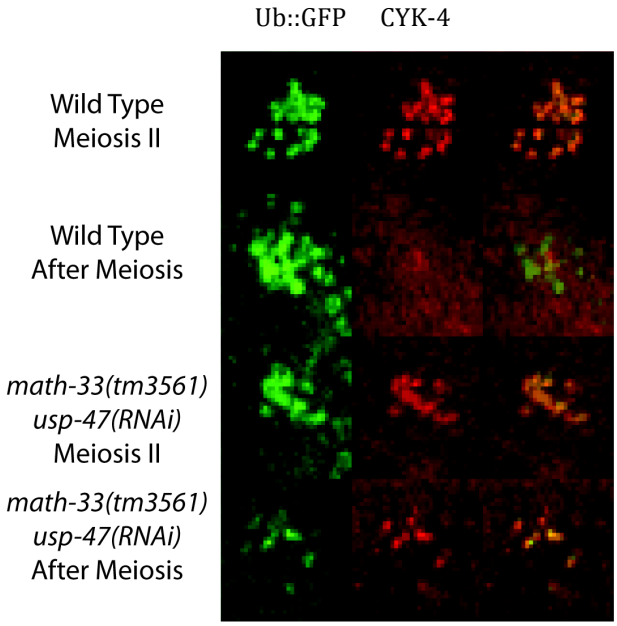
Fibrous body membranous organelles are structures present in mature sperm that enter oocyte cytoplasm upon fertilization by sperm. What is known about the function of these membranous organelles (MOs) is that they are essential for spermatogenesis in part by delivering sperm specific proteins and structures to spermatids (Roberts, Pavalko, & Ward, 1986). The RHO-GEF CYK-4 localizes to MOs, and one presumptive function of this highly concentrated body of CYK-4 is to localize near the posterior cortex where CYK-4 may play a role in breaking symmetry (Jenkins et al., 2006). After fertilization MOs are also ubiquitylated, and are subjected to degradation via an autophagy pathway that is dependent on *atg-7* (Al Rawi et al., 2011). Since MOs co-localize with ubiquitin, I hypothesized that the DUBs MATH-33 and USP-47 may act through MOs to control polarity initiation.

To confirm that CYK-4 co-localizes with ubiquitin to MOs in early embryos I obtained worms expressing ubiquitin::GFP in embryos from the Lynn Boyd lab as well as a rabbit anti-CYK-4 antibody from the Mango Lab (Al Rawi et al., 2011; Jenkins et al., 2006). Upon immunostaining with anti-GFP as well as anti-CYK-4, I found that these two proteins do co-localize to MOs (Appendix Figure A.1). I also observed that CYK-4 localizes to the meiotic spindle microtubules and to the mitotic spindle during anaphase (not shown) which is consistent with its known role as part of the centralspindilin complex (Pavicic-kaltenbrunner, Mishima, & Glotzer, 2007). In wild type, it seems that after or during completion of meiosis II, but before MOs

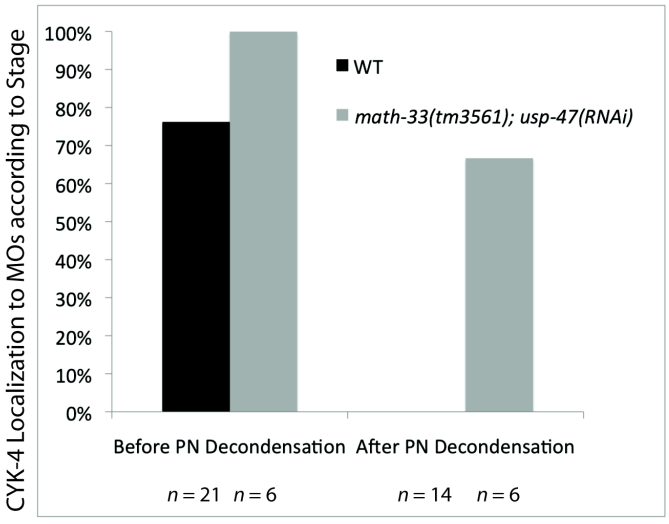
disperse in the embryo, CYK-4 is no longer observed (Appendix Figure A.1), but in *math-33(tm3561); usp-47(RNAi)* CYK-4 remains on MOs and is not removed. CYK-4 persistence on MOs was also observed in *math-33(tm3561)* single mutants with no *usp-47* depletion where I observed many more embryos than in Figure A.2 (not shown). In some cases CYK-4 will remain on MOs up until pronuclear meeting (Appendix 2) or in rare cases up until the 2-cell stage (not shown). Furthermore, MOs that are marked by ubiquitin normally disperse away from the centrosome at the time of polarity establishment (Appendix Figure A.2) (and based on ubiquitin::GFP time-lapses, not shown). However, in *math-33(tm3561); usp-47(RNAi)* embryos MOs remain clustered together for much longer in the cytoplasm. I subjectively judged MOs to be clustered if several (4 or more) were present in the cytoplasm in close proximity (Appendix Figure A.2). Based on the results I tentatively conclude that the distribution of MOs and their association with CYK-4 is affected by loss of the DUBs. Finally, in a preliminary experiment I observed with antibodies whether MOs retained K48 branched ubiquitin chains and K63 branched ubiquitin chains in N2 vs *math-33(tm3561); usp-47(RNAi)* embryos. Both K48 and K63 branched chains were found on MOs as previously reported, and neither species seemed dramatically decreased or increased in DUB mutants (not shown).

Appendix Figure A.1.

A



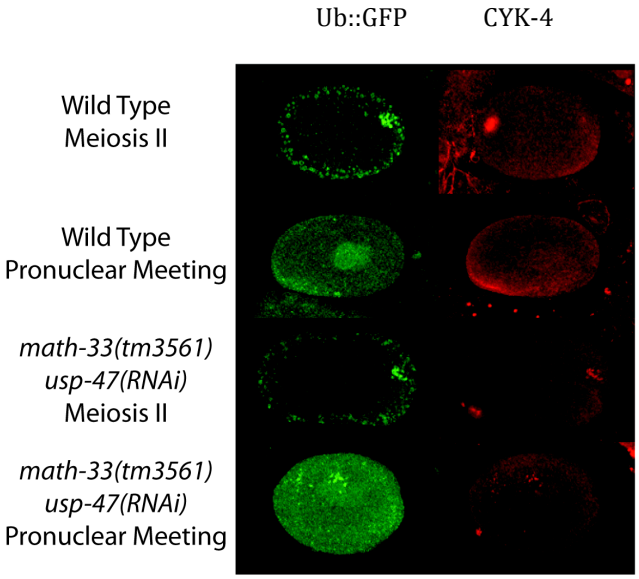
B



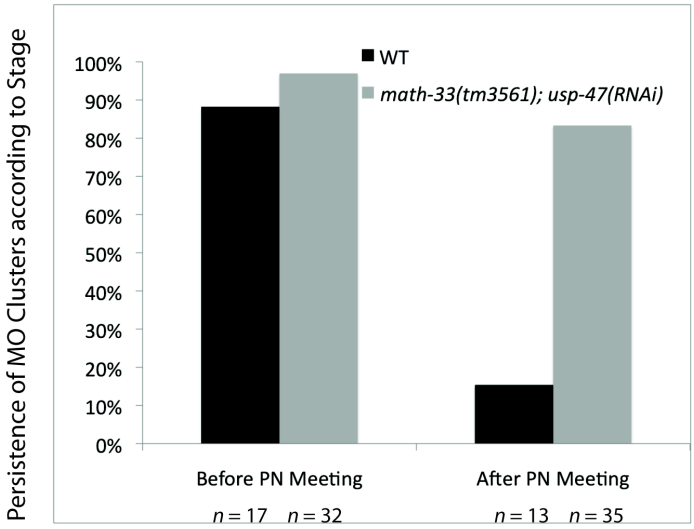
CYK-4 and ubiquitin co-localize on fibrous-body membranous organelles. A) At the end of meiosis, prior to polarization CYK-4 (red) and ubiquitin (green) are enriched on membranous organelles derived from sperm. CYK-4 is normally absent from MOs by the time polarity is initiated, but remains on MOs for longer in *math-33(tm3561)* mutants (not shown) and *math-33(tm3561); usp-47(RNAi).* B) Embryos were observed during or near the end of meiosis before pronuclei decondensed, and then again after the pronuclei have begun to decondensed. 0% (n=14) wild type embryos have CYK-4 present on MOs after pronuclear decondensation, whereas mutants do.

Appendix Figure A.2.

A



B



Membranous organelles remain clustered together instead of scattering in *math-33(tm3561); usp-47(RNAi)* embryos. A) Ubiquitin::GFP (green) and CYK-4 (red) were immunostained in embryos. WT embryos show that membranous organelles scatter throughout the embryo during the polarity establishment phase (also supported by live imaging, not shown). However, in *math-33(tm3561); usp-47(RNAi)* MOs remain clustered near pronuclei and presumably the centrosome after the establishment phase. Sometimes these persistently clustered MOs also contain CYK-4. B) A subjective assessment of the frequency with which MOs clustered either before or after pronuclear meeting.

These observations raise questions about the cause of polarity defects without the DUBs. For example, does CYK-4 persistence on MOs and a change of MO behavior affect the localization of the centrosome in the DUB mutants, or does centrosome localization and function affect the MOs? This question is impossible to answer with the data at hand from my own experiments. In the case that centrosome function is causal, it is clear that the failure of microtubules to function properly may affect where MOs localize in the embryo by causing the scattering defect. However it is not clear how centrosome function would cause CYK-4 to persist on MOs for longer. Indeed it seems that CYK-4 is removed from MOs before MO scattering occurs and CYK-4 removal may be required for scattering to occur. If so, it is possible that the failure of MOs to scatter will block emanating microtubules from the centrosome that would normally contact the cell cortex; this could lead to polarity defects. Since CYK-4 is a component of the centralspindilin complex that binds microtubules it is possible that aggregates of CYK-4 that are near to the centrosome are components of a microtubule binding complex that locally inhibits the centrosome function until the complex is inactivated. In any scenario it is clear that loss of the DUBs affects the MO behavior and composition in a direct way or in a way that is dependent on the function of the centrosome. In the future, more experiments will need to be done to understand the relationship between MOs, CYK-4, and polarity, and the DUBs.

Appendix B. A biochemical approach to find MATH-33 and USP-47 substrates.

Because MATH-33, USP-47, and USP-46 are all putative de-ubiquitylation proteins that may have common substrates I designed experiments with James Moresco of the John Yates lab at the Scripps Institute to find out what those substrates are. Dr. Moresco was interested in MATH-33 because the protein was differentially phosphorylated in a recent screen he had performed. I met Jim at the worm meeting in Los Angeles in 2008, and at the time had already developed an antibody which could be a useful tool to study MATH-33. From that point onward we both participated in experiments in which we immunoprecipitated complexes containing either MATH-33 or USP-47 followed by multi-dimensional separation of peptides, and then mass spectrometry (Wolters, Washburn, & Yates, 2001). During the course of my investigation we performed three specific experiments. During the first experiment Jim immunoprecipitated MATH-33 from healthy adults of either the N2 or *math-33(tm3561)* genotype. Since the antibody I developed could not bind any protein in the putative null *math-33(tm3561),* this genotype served as a negative control. This experiment gave me information about proteins complexed with MATH-33 as well as non-specific binding proteins to subtract from the experiment. The second experiment was one in which I compared GFP immunoprecipitates from MATH-33::GFP and N2 extracts. Since the GFP was expressed maternally under a *pie-1* promoter, this experiment targeted embryos and gonad tissue. During this experiment N2 served as a negative control. In a third and final experiment I immunoprecipitated both MATH-33 and USP-47 in adults using antibodies against each in extracts containing N-ethylmaleimide (+NEM) and

in control extracts (-NEM) as a means to preserve the physical interaction between DUBs and their substrates. NEM covalently modifies free thiol groups, thereby inactivating cysteine catalytic proteases such as MATH-33 and USP-47 (Smyth, 1964), although this activity is promiscuous and could modify thiol groups that are necessary for protein-protein interactions. Therefore, in this experiment, when I compared samples, I expected that those proteins enriched in +NEM extracts were more likely to be DUB substrates that were trapped in complex. With these data in hand I designed a secondary RNAi based screen to identify which, if any of these potential substrates could be involved in polarity. I chose two classes of proteins to screen. First, I chose 54 proteins that were up-regulated in either MATH-33+NEM or USP-47+NEM or both. Second, any gene which was present in all three immunoprecipitation experiments was decidedly interesting, and this group consisted of 15 genes. One of the genes, GMP synthase (GMPS/M106.5) was a useful affirmation that my method was somewhat successful. In previous studies of the mammalian homolog of MATH-33, USP7, GMPS was found to be a protein that can stably complex with USP7 and also may positively regulate USP7 enzymatic activity (Faesen et al., 2011; Sarkari et al., 2009). After choosing genes, I planned deplete target genes in several genetic backgrounds; N2, *math-33(tm3561), par-2(it5ts),* and *usp-47(tm4950).* My assumption is that depleting a substrate of MATH-33 or USP-47 would have the same effect as loss of MATH-33 and USP-47 because they are positive regulators of protein levels. Therefore, I screened for both low level embryonic lethality and polarity defects similar to those observed in *math-33* or *math-33; usp-47(RNAi)*.

After performing the enhancer screen, 14 genes were selected for plate counting based on increases in embryonic lethality. 11/14 showed an increase in embryonic lethality in at least one of *par-2(ts), math-33,* or *usp-47* relative to N2. The remaining 3/14 RNAi candidates were screened because they caused high embryonic lethality in all genotypes and could possibly have polarity phenotypes regardless of genetic background.

Table A.1.

TRIPLE OVERLAP MATH-33 These proteins overlap in all 3 MATH-33 experiments		
Gene ID	**gene name**	**USP-47**
C06B8.8	*rpl-38*	yes
C25A1.6	*nra-4*	no
C34G6.7a	*stam-1*	no
C44C1.4a	*vps-45*	no
C56G2.1a	*n/a*	no
E01G4.4	*pqn-27*	no
F25H5.1b	*lim-9*	no
K07A1.8	*ile-1*	no
K08E3.1	*tyr-2*	no
R04A9.4	*ife-2*	no
T11B7.4c	*alp-1*	no
T16G12.5	*ekl-6*	no
Y105E8A.3	*n/a*	no
Y57G11C.3b	*6PGL*	no
ZK836.2	*n/a*	no

Proteins which co-immunoprecipitated with MATH-33 or MATH-33::GFP in three independent experiments. Column 3 indicates whether the protein was found to co-immunoprecipitate in a single anti-USP-47 experiment. These genes were selected for RNAi follow-up screening.

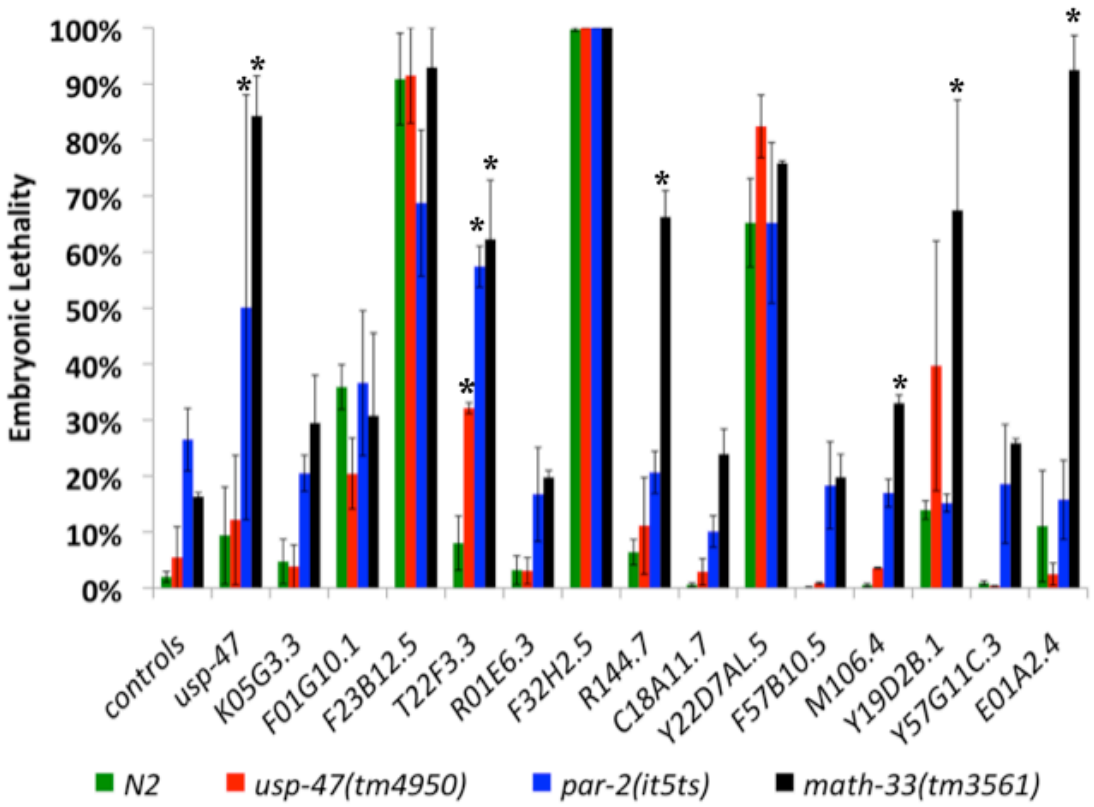
Table A.2.

Gene ID	Gene name	Gene ID	Gene name
C04F12.4	*rpl-14*	*K12F2.1*	*myo-3*
c06a1.1	*cdc-48.1*	*llc1.3*	*dld-1*
c18a11.7	*dim-1*	*M03F4.2a*	*act-4*
C44E4.4	*C44E4.4*	*m106.4*	*M106.4*
C47E8.5	*daf-21*	*r01e6.3*	*cah-4*
C49H3.11	*rps-2*	*R03G5.1a*	*eef-1A.2*
C52E4.4	*rpt-1*	*R06C7.10*	*myo-1*
f01g10.1	*tkt-1*	*r144.7*	*larp-1*
F01G4.6a	*F01G4.6*	*rpt-1*	*rpt-1*
f09f7.2	*mlc-3*	*T04A8.6*	*T04A8.6*
F10G7.2	*tsn-1*	*t05g5.6*	*ech-6*
F11C3.3	*unc-54*	*T07A9.11*	*rps-24*
F20D1.4	*plp-2*	*t17h7.1*	*T17H7.1*
f23b12.5	*dlat-1*	*t22f3.3*	*T22F3.3*
F23F12.6	*rpt-3*	*T23E7.2e*	*T23E7.2*
f25b5.6	*F25B5.6*	*t25c12.3*	*T25C12.3*
f25h2.11	*tct-1*	*t27e9.7*	*abcf-2*
F25H5.3b	*pyk-1*	*W08E3.3*	*tag-210*
F25H5.4	*n/a*	*y19d2b.1*	*Y19D2B.1*
F31E3.5	*eef-1A.1*	*y22d7al.5*	*hsp-60*
f32h2.5	*fasn-1*	*Y24D9A.4c*	*rpl-7A*
F42C5.8	*rps-8*	*Y37E3.9*	*phb-1*
F45E4.2	*plp-1*	***Y39B6A.20***	*asp-1*
F46H5.3a	*F46H5.3*	*y41e3.4*	*qars-1*
F53A3.3	*n/a*	*y55f3ar.3*	*cct-8*
f54e2.3	*ketn-1*	*y66h1b.2*	*fln-1*
f57b10.5	*F57B10.5*	*y71h2am.23*	*tufm-1*
F59B8.2	*idh-1*	*Y73B3A.18*	*Y73B3A.18*
K02F2.2	*ahcy-1*	*Y73B6BL.6b*	*sqd-1*
K04D7.3	*gta-1*	*zk822.1*	*ZK822.1*
k05g3.3	*cah-3*	*zk829.4*	*gdh-1*

Proteins that co-immunoprecipitate with both MATH-33 and USP-47 from worm extracts, and that are enriched in the presence of NEM. These genes were selected for follow-up RNAi screening based on the criteria that they were enriched in either MATH-33 and/or USP-47 immunoprecipitates when NEM was present.

Genes for which RNAi clones were not available are listed in bold.

Appendix Figure B.1



Final candidate embryonic lethality enhancers for proteins which co-immunoprecipitate with USP-47 or MATH-33. A few candidates, such as *T22F3.3, R144.7, Y19D2B.2, or E01A2.4* showed significant enhancing effects.

Of these 14 RNAi experiments, three showed high embryonic lethality on their own and several have what appear to be weak enhancing effects on one or more allele. However, four enhancers were found to be statistically significant by a T test $p < 0.05$. Two genes, *R144.7* and *E01A2.4* were *math-33(tm3561)* specific enhancers. One gene, *Y19D2B.1* may be a specific enhancer of *math-33(tm3561)* and *usp-47(tm4950)* although only *math-33* genetic enhancement was statistically significant. Finally one gene, *T22F3.3* was an enhancers of both DUB mutants as well as *par-2(it5ts).* I next tested whether the depletions in these genetic backgrounds showed polarity defects consistent with a role in early embryonic polarity. Examination of all the relevant RNAi/mutant combinations showed no apparent polarity defects at the one and two-cell stages. Therefore, it is likely that these genetic enhancers increase embryonic lethality in pathways not directly impinging on polarity in the one-cell embryo.

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